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Posttraumatic stress disorder (PTSD) is a syndrome of symptoms indicative of emotional dysfunction, which develop after exposure to life-threatening events. Prevalent symptoms are exaggerated fear and anxiety, which become particularly intense during exposure to situations reminiscent of the traumatic events that precipitated the disease. The amygdala is a key component of the brain's neuronal network that determines the emotional significance of external events. Despite the central role of the amygdala in emotional behavior, little is known about the impact of stress on the amygdala's function. Clinical evidence indicates that norepinephrine and serotonin may participate in modulating the synaptic plasticity phenomena that result in the memory of frightening events in PTSD. Our data indicate that the modulatory effects of norepinephrine and serotonin receptors on synaptic transmission, neuroplasticity and calcium homeostasis are altered in traumatically stressed rat amygdala. The results of this study may aid in the development of new strategies aimed at modifying and preventing the formation of traumatic memory, and thus could be useful for the treatment of combat PTSD in veterans.

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INTRODUCTION:

Intense or chronic stress can have long-lasting consequences on an individual's health, and can be the cause of debilitating mental illnesses. A very common mental illness induced by traumatic stress is posttraumatic stress disorder (PTSD). PTSD is a syndrome of symptoms indicative of emotional dysfunction, which develop after exposure to life-threatening events, or very stressful situations of different nature. Prevalent symptoms are exaggerated fear and anxiety, which become particularly intense during exposure to situations reminiscent of the traumatic events that precipitated the disease. PTSD is a psychiatric disorder of considerable prevalence and morbidity and can affect persons of any age and ethnic or socioeconomic background. It is, unfortunately, a far too common result of participation in wars. In addition, epidemiological studies have suggested that the prevalence of PTSD is even higher in inner city communities exposed to compound community trauma. It is imperative, therefore, to understand the neurobiological mechanisms by which exposure to traumatic stress leads to PTSD in order to foster the development of new therapeutic strategies for the prevention and treatment of stress-related affective disorders such as PTSD.

Clinical evidence indicates that certain stress-related affective disorders such as PTSD are associated with changes in the amygdala's excitability. The amygdala is a key component of the brain's neuronal network that determines the emotional significance of external events (LeDoux, 1992; Davis, 1994; Breiter et al., 1996; Schneider et al., 1997; LaBar et al., 1998; Buchel et al., 1998; Whalen et al., 1998; Baird et al., 1999; Davidson et al., 1999; Davidson and Slagter, 2000; Buchel and Dolan, 2000). Via efferent pathways to the hypothalamus, the amygdala can trigger the neuroendocrine cascades that are part of the stress response (Habib et al., 2001; Pitkänen, 2000; Davis, 1992; Davis et al., 1994) and via reciprocal connections with the cerebral cortex and limbic structures, it modulates the orchestration of the behavioral response (Goldstein et al., 1996; Pitkänen, 2000). Despite the central role of the amygdala in emotional behavior, little is known about the impact of stress on amygdala function.

The amygdala is a brain region critical brain critical to the consolidation of emotional memory; thus, in fear conditioning, an experimental paradigm that shares characteristics of anxiety disorders and PTSD, the amygdala mediates the learning experience and is likely to be the site of memory storage. Evidence supporting this view is as follows: 1) Fear-conditioning is associated with a strong activation of the amygdala in both rats and humans, 2) Parallel to the development of the conditioned fear-behavior, there is a long-lasting potentiation of evoked field potentials or synaptic currents of amygdala neurons. 3) Amygdala lesions severely impair or block both the acquisition and expression of conditioned fear. In other types of unconditioned emotional experiences, the amygdala is believed to play a central role in modulating the consolidation of emotional memories in the cerebral cortex, as well as in modulating the function of other limbic structures involved in memory formation (Kim et al., 2001).

Increased release of norepinephrine and serotonin in the amygdala is associated with behaviors that are typically seen during states of fear, suggesting that norepinephrine

and serotonin may play a role in amygdala circuits mediating fear responses (Servatius et al., 1995). It has been speculated that norepinephrine and serotonin may also participate in the synaptic plasticity phenomena that result in the memory of frightening events and also in PTSD. The short-term effects and long-term consequences of stress-induced excessive norepinephrine and serotonin release on amygdala physiology are unknown.

The studies proposed in this grant focused on the roles of norepinephrine and serotonin receptors on activity-dependent neuroplasticity and calcium signaling in amygdala slice preparations from traumatically stressed and control rats. The hypothesis we have raised is that the modulatory effects of norepinephrine and serotonin receptors on synaptic transmission, neuroplasticity and calcium homeostasis are altered in traumatically stressed rat amygdala. The results of this study may aid in the development of new strategies aimed at modifying and preventing the formation of traumatic memory, and thus could be useful for the treatment of combat PTSD in veterans.

BODY:

In the five years of this project we have concentrated our efforts on identifying the neurobiological alterations in amygdala physiology and function induced by traumatic stress. Specifically, we have discovered the mechanisms underlying the stress-induced hyperexcitability in the amygdala. We have found that stress impairs the function of a specific subtype of adrenergic receptors (the α_{1A} adrenoceptor) that mediates the actions of noradrenaline (a neurotransmitter that plays a central role in the stress response). Activation of these receptors by noradrenaline facilitates inhibitory activity in the amygdala, preventing over-excitation and suppressing synaptic neuronal plasticity that takes place during memory formation. The stress-induced impairment in the function of these receptors will therefore result in neuronal hyperexcitability and hyper-responsiveness in the amygdala, and will facilitate the "registration" of memories associated with emotionally significant events.

These findings provide, for the first time, direct evidence that stress causes impairment in the modulation of inhibitory activity in the brain, and thus offer an important insight into the possible mechanisms underlying the hyperexcitability and hyperresponsiveness of the amygdala in certain stress-related mental illnesses such as PTSD, as well as in the stress-induced exacerbation of seizure activity in epileptic patients. These important findings have been recentely published in ***Neuropsychopharmacology* 29 (1): 45-58, 2004**. In addition, the abstract of this work was presented at the ***Faculty Senate Research Day and Graduate Student Colloquia* (From Bench to Bedside and Battlefield: Translational Research at the Nation's Medical School)** at USUHS, Bethesda, MD. This poster was selected from more than 150 abstracts submitted by researchers from the Uniformed Services University and its affiliates to be presented at the ***PRESIDENT'S POSTER SESSION.*** The selection was based on the high-impact military and civilian biomedical research conducted at USUHS and its affiliates.

The same abstract was submitted for presentation at the Society for Neuroscience meeting in New Orleans (Novemeber, 2003). Again, our abstract was one of 600 (among more than 15,000 submissions from all over the world) requested by the **Public Information Committee** for inclusion in the **Annual Meeting Press Book** as a lay-language summary. These summaries are used to set up press interviews with scientists whose work is found to have a major impact on a specific research field.

Detailed description of key research accomplishments

An animal model for studying stress-related disorders

Traumatic stress can have long-lasting consequences for an individual and can result in affective disorders such as PTSD. No preventive treatment currently exists for PTSD. The development of an animal model is critical for the study of therapeutic and prophylactic treatments. Historically, effective therapeutic treatments have been developed for diseases only if adequate animal models were available. We have successfully established and tested the inescapable tail-shock model of stress in rats and verified that short and long-lasting behavioral and physiological alterations, known to be mediated by the amygdala, resulted from applying the inescapable tail-shock stress paradigm to the animals studied. The behavioral and neurobiological alterations induced by this rat model are similar to those found in PTSD patients (Table 1 below).

In our model, stress exposure consists of a two-hour per day session of immobilization and tail-shocks, for three consecutives days. The animals are restrained in a plexiglas tube, and 40 electric shocks (2 mA, 3 s duration) are applied at varying intervals (140 to 180 s). This stress protocol was adapted from the "learned helplessness" paradigm in which animals undergo an aversive experience under conditions in which they cannot perform any adaptive response (Seligman and Maier, 1967; Maier and Seligman, 1976). We stress the rats for three consecutive days because it has been previously demonstrated that repeated immobilization and tail-shock stress sessions for three days is more effective that a single stress session in producing physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight. Further exposure to stress does not appear to result in greater physiological and behavioral changes.

In our study, body weight of the stressed group was 45 ± 2.1 g ($n = 21$) before the first stress session, and 54.5 ± 2.8 g ($n = 18$) after the last stress session (Figure 1). Control rats, weighted at the same time of the day before the first stress session, were 46 ± 1.7 g ($n = 24$), and 63 ± 2.1 g ($n = 21$) after the last stress session (Figure 1). The difference in body weight between stressed and control rats after the last stress session was statistically significant ($p < 0.05$) and stressed rats continued to display reduced body weight gain for as long as body weight was monitored (up to 10 days after stressor cessation).

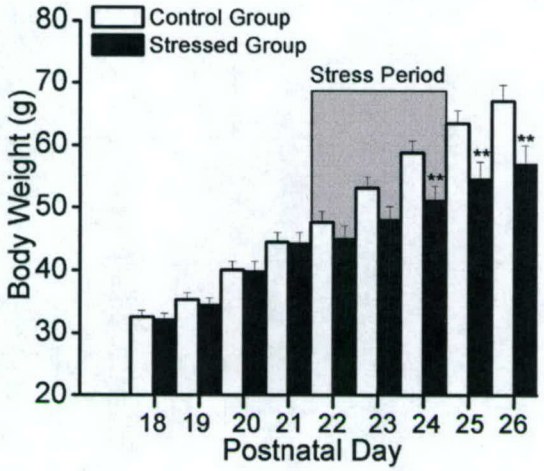

Figure 1. Restrain/tail-shock stress reduces body weight gain. Exposure to stress on postnatal days (PDN) 22, 23 and 24 reduced body weigh gain. Body weight difference between control and stressed rats was statistically significant after the first day of stress (**$p < 0.01$). Data on PDN 26 are from rats that were not used for electrophysiological experiments. Sample sizes range from 12 (PDN 26) to 24 rats.

We have also assessed the effectiveness of our tail-shock model of stress in rats by measuring behavioral response to acoustic startle, and circulating corticosterone levels. The group of rats receiving restraint and tail shock had potentiated startle responses from the 4^{th} day through the 10^{th} day following stress exposure (26.7 to 66.7% greater than the group receiving no treatment, $n = 26$, $p<0.05$). Tail blood samples were collected and assayed for corticosterone levels at three time points during the study: a) immediately before stress, b) after the first and c) after the third day of stress. All stressed groups displayed elevated basal corticosterone levels (41 ±4.2% and 53 ±43.8%, $n = 24$, $p<0.05$) confirming previously published studies published by Servatius et al., 1995. Thus, our results of the inescapable tail shock paradigm as a model of PTSD are consistent with those in the literature (Servatius et al, 1995) and indicate that we have successfully established and tested the inescapable tail-shock model of stress in rats.

Table 1. Comparison of Symptoms of PTSD in Humans to Dysfunction Related to Stress in Rats

PTSD in Humans	Inescapable tail-shock model of stress in rats
Weight loss	Suppressed feeding and body weight loss
Difficulty falling or staying asleep, nightmares	Altered sleep patterns
Psychomotor numbness	Persistent behavioral abnormalities i.e. suppressed open-field activity, longer hanging wire latencies
Poor concentration; memory deficits	Deficits in escape/avoidance learning and learning of an appetitive task
Hypervigilance and/or exaggerated startle reponse	Exaggerated startle
Hyperresponsiveness of the noradrenergic system	Hyperresponsiveness of the noradrenergic system

Stress impairs α_{1A} adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala.

Intense or chronic stress can produce pathophysiological alterations in the systems involved in the stress response. The amygdala is a key component of the brain's neuronal network that processes and assigns emotional value to life's experiences, consolidates the memory of emotionally significant events, and organizes the behavioral response to these events. Clinical evidence indicates that certain stress-related affective disorders are associated with changes in amygdala excitability, implicating possible dysfunction of the GABAergic system. An integral component of the stress response is the activation of the noradrenergic system. Norepinephrine (NE), acting via α_1 adrenoceptors, modulates GABAergic inhibition. Despite evidence for stress-related impairments in both α_1 adrenoceptor function and GABAergic transmission, as well evidence that α_1 adrenoceptors mediate the noradrenergic modulation of GABAergic transmission, an association between stress, impaired function of α_1 adrenoceptors, and pathophysiological alterations in GABAergic inhibition has not been previously considered.

Recently we investigated whether NE modulates GABAergic transmission in the basolateral nucleus of the amygdala (BLA), and if so, whether noradrenergic modulation of GABAergic transmission was altered by exposure to traumatic stress. We studied the BLA because this amygdala region is heavily involved in the processing of emotional experiences, as it receives both direct and indirect thalamic and cortical inputs and it is extensively interconnected with the prefrontal/frontal cortex and the hippocampus (Pitkänen, 2000). Furthermore, it appears that the BLA selectively (among the different amygdala nuclei) modulates the consolidation of emotional memories. Our results show that NE facilitates spontaneous, evoked, and action potential-independent, quantal GABA

release in the BLA via α_{1A} adrenoceptors, and that these effects of NE are virtually absent in stressed rats.

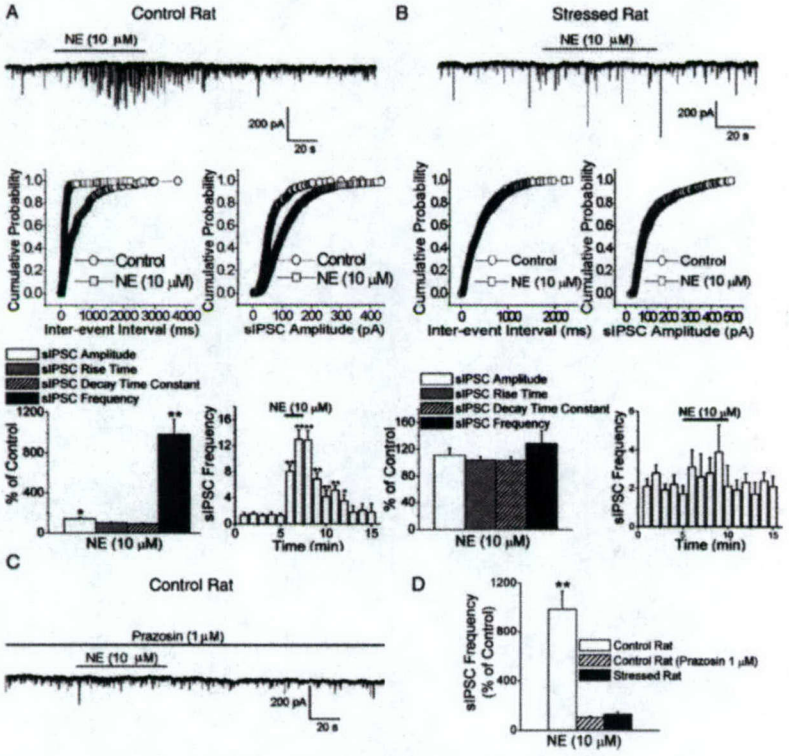

Figure 2. Activation of α_1 adrenoceptors increases tonic inhibition of BLA pyramidal neurons in control rats, but not in stressed rats. (A) Top trace: Effects of NE on spontaneous IPSCs recorded from a BLA pyramidal cell of a control rat. (B) Top trace: sIPSCs recorded from a BLA pyramidal cell of a stressed rat. Middle graphs: Cumulative probability plots of inter-event intervals and amplitude of sIPSC. Bottom graphs: (C) Prazosin prevented the NE-induced increase of sIPSCs. (D) Bar graph shows the effects of NE on the mean frequency of sIPSCs recorded from control rats, and stressed rats. *$p<0.05$.

Noradrenergic modulation of spontaneous IPSCs

To investigate whether NE modulates GABAergic transmission in the BLA, and whether stress alters this modulation, we first examined the effects of NE on action-potential dependent, spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from BLA pyramidal neurons, in control and stressed rats. Spontaneous IPSCs were recorded at a holding potential of –70 mV, and in the presence of D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM) and yohimbine (20 μM) to block NMDA, AMPA/kainate, β and α_2 receptors, respectively.

In control rats, the mean frequency of sIPSCs recorded in the soma of BLA pyramidal neurons was 3.1 ± 1.6 Hz (n = 21). Bath application of bicuculline (10 μM) eliminated sIPSCs, confirming that they were mediated by $GABA_A$ receptors. NE (10 μM) caused a significant increase of the mean sIPSC frequency (984.3.9 ± 148.2% of the control values, n = 21, $p<0.01$; Fig. 2A) and amplitude (144.0 ± 12.8% of the control values, n = 21, $p<0.05$; Fig. 2A) that persisted throughout the application of NE and was completely reversed after removal of the agonist. These effects of NE were not accompanied by any significant change in the rise time or decay time constant of sIPSCs (Fig. 2A), and were blocked by the α_1 adrenoceptor antagonist prazosin (1 μM, Fig. 2C) confirming that NE was acting via α_1 adrenergic receptors. In stressed rats, the mean frequency of spontaneous IPSCs was 2.6 ± 2.3 Hz. NE (10 μM) had no significant effect on the frequency or amplitude of sIPSCs. Thus, in the presence of NE (10 μM), the frequency of sIPSCs was 128.9 ± 19.2% and the amplitude was 111.4 ± 10.2% of control values (n = 19, Fig. 2B). In addition, bath perfusion of NE (10 μM) caused no significant changes in the kinetics of these currents (rise time and decay time constant of sIPSCs; Fig. 2B).

To identify the subtype of α_1 adrenoceptors involved in the effects of NE on control rats, we first applied NE (10 μM) in the additional presence of CEC (10 μM) and BMY 7378 (300 nM) to block α_{1B} and α_{1D} adrenoceptors. There was no significant attenuation of the effects of NE in the presence of these antagonists. Thus, NE increased the frequency of sIPSCs from 2.8 ± 2.4 Hz to 27.1 ± 7.9 Hz ($p<0.01$, n = 6), and the amplitude of sIPSCs to 154 ± 11.3% of control values ($p<0.05$, n = 6). Next, we examined the effects of the specific α_{1A} adrenoceptor agonist A61603. In control rats, A61603 (1 μM) increased the frequency and amplitude of sIPSC to 1034 ± 158.6 % and 162 ± 14.2% of control values, respectively ($p<0.01$, n = 16; Fig. 3A).

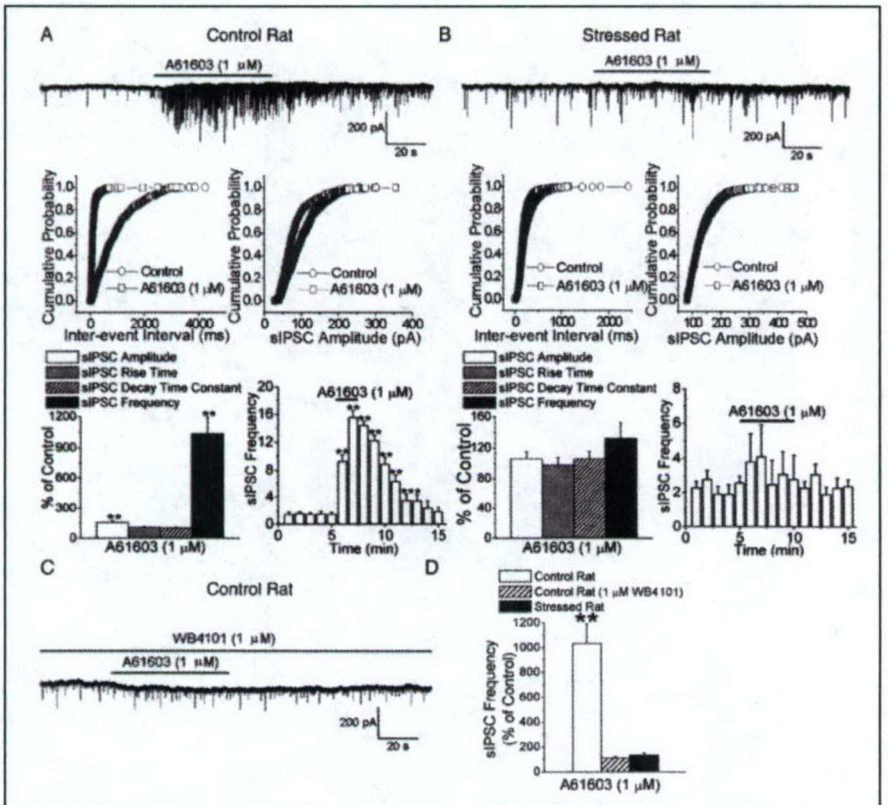

Figure 3. Activation of α_{1A} adrenoceptors increases tonic inhibition of BLA pyramidal neurons in control rats, but not in stressed rats. (A) Top trace: Effects of A61603 on spontaneous IPSCs recorded from a BLA pyramidal cell of a control rat. (B) Top trace: sIPSCs recorded from a BLA pyramidal cell of a stressed rat. Middle graphs: Cumulative probability plots of inter-event intervals and amplitude of sIPSC. Bottom graphs: (C) WB4101 prevented the NE-induced increase of sIPSCs. (D) Bar graph shows the effects of A61603 on the mean frequency of sIPSCs recorded from control rats, and stressed rats. *p<0.05.

There were no effects on the rise time or decay time constant of sIPSCs (Fig. 3A). In stressed rats, A61603 had no significant effect (Fig. 3B). Thus, in the presence of 1 μM A61603 the frequency of sIPSCs was 132 ± 21% and the amplitude of sIPSCs was 106 ± 8.8% of control values (n = 18, Fig. 3B). The effects of A61603 on sIPSCs in control rats were blocked by the selective α_{1A} adrenoceptor antagonist WB4101 (1 μM, Fig.3C and D).

Taken together these results suggest that NE enhances tonic inhibition of pyramidal cells in the BLA, by inducing a massive increase in action potential-dependent spontaneous release of GABA via α_{1A} adrenoceptors, and stress impairs this function of α_{1A} adrenoceptors.

Noradrenergic modulation of evoked IPSCs

It has been shown previously that NE reduces evoked inhibitory transmission in the hippocampus via α adrenoceptors. More recently, in the sensorimotor cortex, it was found that NE actually has a small facilitatory effect on evoked IPSCs, which is detected when $GABA_B$ receptors are blocked. To determine the effects of NE on evoked inhibitory transmission in the BLA we applied 10 μM NE while recording evoked IPSCs (eIPSCs) in control rats. In the absence of a $GABA_B$ receptor antagonist, NE (10 μM) reduced the amplitude of eIPSCs to 48.2 ± 10.3% of control levels ($p<0.01$, n=8). However, in the presence of SCH50911 (20 μM), a specific antagonist of the $GABA_B$ receptors, NE enhanced the amplitude of eIPSCs to 162.4 ± 9.3% of control, $p<0.01$, n = 10; Fig. 4A) without affecting the rise time and decay time constant of the eIPSCs (Fig. 4A). Similar effects were obtained when α_{1A} adrenoceptors were activated by application of 1 μM A61603 (Fig 4C). Thus, A61603 (1 μM) increased the amplitude of eIPSCs to 159.4 ± 10.7% of control ($p<0.01$, n = 8, Fig. 4C) without affecting the kinetics of the eIPSCs (Fig. 4C). The effects of the drugs were reversible. In stressed rats, neither NE nor A61603 had a significant effect on the amplitude, rise time and decay time constant of eIPSCs (Fig. 4B and D). In the presence of NE (10 μM), eIPSC amplitude was 109 ± 8.2% of the control (n = 11), and in the presence of A61603 the amplitude of the evoked IPSCs was 103 ± 7.4 % of the control (n = 10). These results suggest that 1) NE facilitates evoked GABAergic transmission via α_{1A} adrenergic receptors, 2) this facilitatory effect is masked due to activation of presynaptic $GABA_B$ autoreceptors following the NE-induced enhancement of spontaneous GABA release, and 3) stress blocks the facilitatory effect of NE on evoked GABA release.

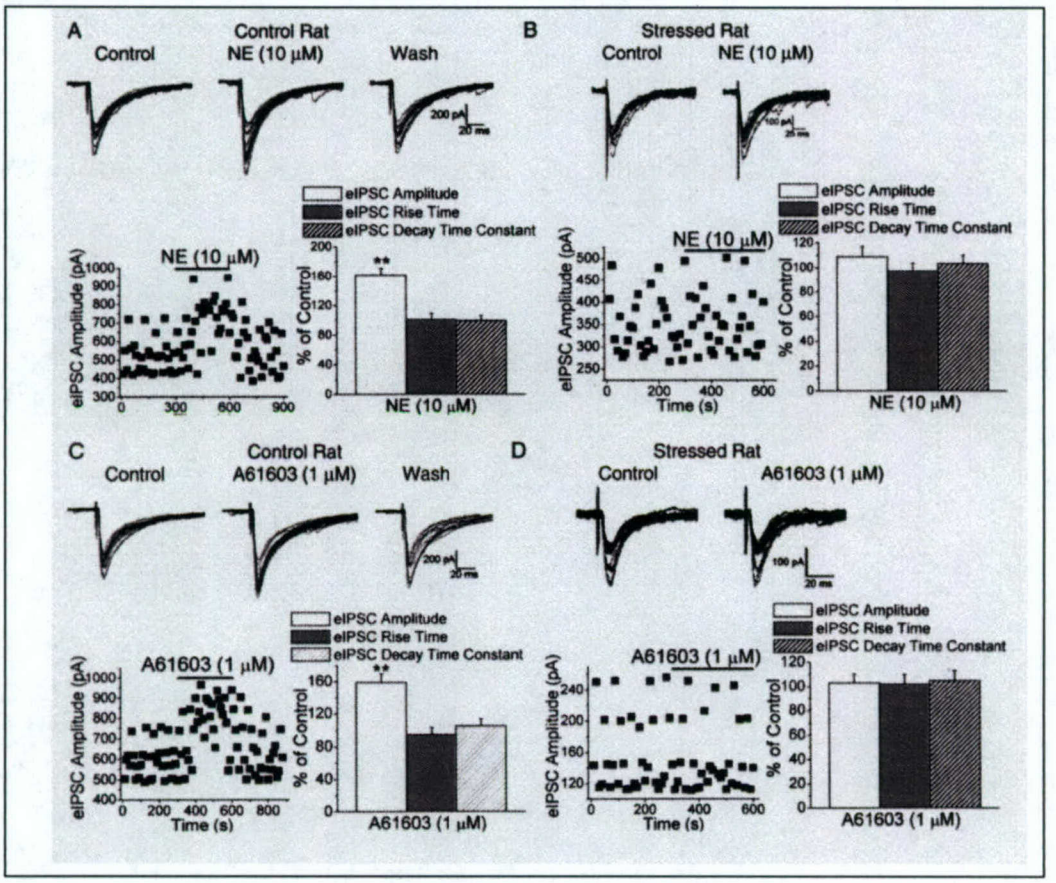

Figure 4. In the presence of a $GABA_B$ receptor antagonist, activation of α_{1A} adrenoceptors increases the amplitude of evoked IPSCs in control rats, but not in stressed rats.

(A)Top traces: Evoked IPSCs (eIPSCs) recorded from a BLA pyramidal cell of a control rat. In addition to D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM) and yohimbine (20 μM), the slice medium also contains 20 μM SCH50911. Norepinephrine increased the amplitude of the eIPSCs, without affecting their kinetics. Bottom graphs: The plot shows the time-course of the NE effect on eIPSC amplitude (same cell as in the top traces). The bar graph shows the effect of NE on the amplitude and kinetics of eIPSCs. (B) Data similar to those shown in (A), but from stressed rats. (C) In control rats, the α_{1A} agonist A61603 produced similar effects to those of NE. Top traces and bottom left plot show data from the same cell. (D) In stressed rats, A61603 had no significant effects on eIPSCs.

Noradrenergic modulation of mIPSCs

The enhancement of eIPSCs and action-potential dependent sIPSCs by NE could be due to a depolarizing effect via activation of somatodendritic α_{1A} adrenoceptors on GABAergic neurons, and/or due to a direct effect at GABAergic terminals. To determine whether, in the BLA, NE modulates GABA release by a direct effect on GABAergic

terminals, we tested the effects of NE on miniature IPSCs (mIPSCs), which do not depend on presynaptic invasion of action potentials or Ca^{2+} influx. Miniature IPSCs were recorded in medium containing D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), yohimbine (20 μM) and TTX (1 μM). In the absence of NE, the frequency of mIPSCs was 0.68 ± 0.32 Hz and their amplitude was 114.0 ± 12 pA (n =10). NE (10 μM) increased the frequency of mIPSCs to 182.3 ± 9.6 % of control ($p<0.01$, n = 10; Fig. 5A). The amplitude, rise time and decay time constant of the mIPSCs were not significantly affected by 10 μM NE (Fig. 5A). Similar effects were observed after application of the α_{1A} specific agonist A61603 (Fig. 5C). A61603 (1 μM) increased the frequency of mIPSCs from 0.71 ±0.24 to 1.28 ± 0.31 (178 ± 12.4% of control, $p<0.01$, n = 9; Fig. 5C). The amplitude and kinetics of mIPSCs were not affected by A61603 (Fig 5C). In stressed rats, neither NE (10 μM) nor A61603 (1 μM) produced a significant effect on mIPSCs frequency, amplitude or kinetics (Fig. 5B and C). Thus, the frequency of mIPSCs was 0.68 ± 0.25 Hz and 0.64 ± 0.34 Hz before and after application of NE, respectively (n = 10), and 0.72 ± 0.27 Hz and 0.63 ± 0.31 Hz in the presence and absence of 1 μM A61603, respectively (n = 8). These results suggest that 1) NE facilitates GABA release by a direct effect on GABAergic terminals, and 2) this mechanism of noradrenergic facilitation of GABA release is impaired by stress.

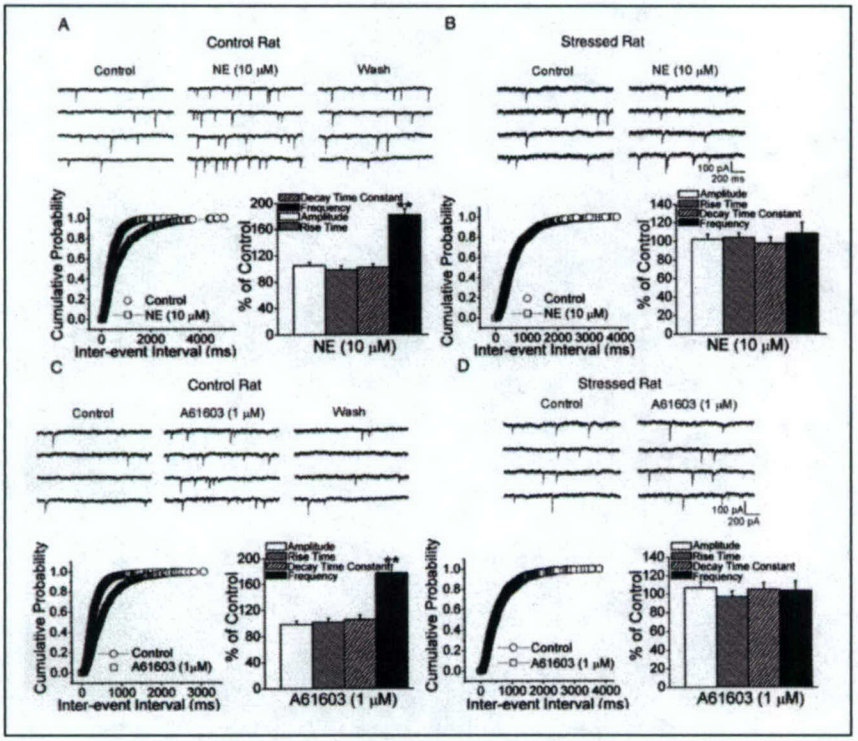

Figure 5. Activation of α_{1A} adrenoceptors increases the frequency of mIPSCs in control rats, but not in stressed rats. (A) Top traces: mIPSCs recorded from a BLA pyramidal neuron of a control rat. NE (10 uM) increased the frequency of mIPSCs. Bottom graph: Left panel shows the cumulative probability plots of inter-event intervals of mIPSCs in control conditions and during application of NE. Bar graph shows the

effect of NE on the amplitude, kinetics, and frequency of mIPSCs **$p<0.01$. (B) Similar data to those shown in (A), but from stressed rats. NE had no significant effect on mIPSCs. (C) In control rats, the α_{1A} antagonist A61603 had similar effects to those induced by NE. (D) A61603 had no significant effects on mIPSCs recorded from BLA pyramidal cells of stressed rats. Bar graphs show pooled data from 8 cells.

Facilitation of GABAergic transmission by α_{1A} adrenoceptors is mediated by phospholipase C

Studies in other brain regions or cell types have shown that α_1 adrenoceptors are coupled to phospholipase C (PLC) via a G-protein, and can increase intracellular calcium by mobilizing Ca^{2+} from intracellular stores, as well as by increasing Ca^{2+} influx. However, certain effects of α_{1A} activation involve signaling pathways that are independent of PLC activation and intracellular Ca^{2+} rise. To determine whether the α_{1A} adrenoceptor-mediated facilitation of GABA release in the BLA involves activation of PLC, we examined whether the effects of NE on GABAergic transmission are blocked by a PLC inhibitor. In control rats, NE (10 μM) or A61603 (1 μM) enhanced the frequency and amplitude of sIPSCs in the presence of U73343 (20 μM), the inactive isomer of the PLC inhibitor U73122, but had no effects in the presence of 20 μM U73122 (Fig. 6). Thus, in the presence of U73343, NE increased the frequency of sIPSCs to 1022.8 ± 105.3% of control levels ($p<0.01$, $n = 8$; Fig. 6) and increased the amplitude of sIPSCs to 161 ± 11.7% of control levels ($p<0.01$, $n = 6$; Fig 6). A61603 (1 μM) increased the frequency of sIPSCs to 978.1 ± 102.1% ($p<0.01$, $n = 8$; Fig. 6), and increased the amplitude of sIPSCs to154 ± 12.3% of control levels ($p<0.01$, $n = 8$; Fig 6).
In contrast, in the presence of U73122 (20 μM) NE (10 μM) and A61603 (1 μM) failed to induce any significant changes in the frequency and amplitude of sIPSCs. Similarly, the effects of NE (10 μM) on the amplitude of eIPSCs, as well as on the frequency of mIPSCs were blocked by 20 μM U73122 (not shown).

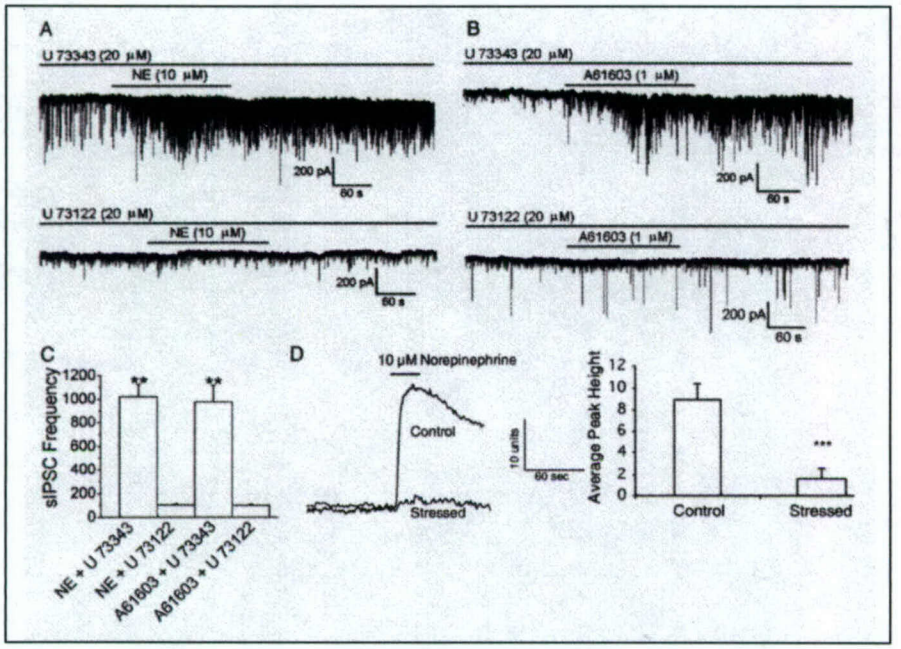

Figure 6. α_{1A} adrenoceptors in the BLA are coupled to phospholipase C. (A) and (B). Spontaneous IPSCs (sIPSCs) recorded from BLA pyramidal neurons. NE (A) or A61603 (B) increased the frequency and amplitude of sIPSCs in the presence of the inactive isomer of a PLC inhibitor (U73343), but had no effect in the presence of the PLC inhibitor U73122. The slice medium contains D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM) and yohimbine (20 μM). (C) Bar graphs showing the effects of NE (10 μM) or A61603 (1 μM) on the frequency of sIPSCs, in the presence of U73343 or U73122. Pooled data from 8 neurons.

Next, we examined whether activation of α_{1A} adrenoceptors by NE, in the BLA, enhances the concentration of intracellular Ca^{2+}. In Fura-2AM-loaded slices, 10 μM NE was applied for 30 s, in the presence of bicuculline (10 μM), SCH50911 (5 μM), CNQX (50 μM), D-AP5 (50 μM), propranolol (10 μM), and yohimbine (20 μM). Intracellular Ca^{2+} concentration was significantly enhanced in slices from control rats, whereas NE had no significant effect in slices from stressed rats (Fig. 6D). Thus, in control rats, NE (10 μM) produced a 4.21 ± 1.47-fold change relative to baseline (change in ratios of fluorescence intensity, $p<0.01$, $n = 14$; Fig. 6D), while in stressed rats there was only a 1.70 ± 0.94-fold change ($n = 14$; Fig. 6D). Similar results were obtained when α_{1A} adrenoceptors were activated by application of A61603 (1 μM). In control rats, A61603 (1 μM) induced a significant increase in intracellular Ca^{2+} (4.68 ± 0.17-fold change relative to baseline, $p<0.05$, $n = 16$), while in the stressed rats A61603 failed to cause a significant enhancement (0.198 ± 0.081-fold change, $n = 16$).

Stress blocks α_{1A} adrenoceptor-mediated suppression of BLA field potentials.

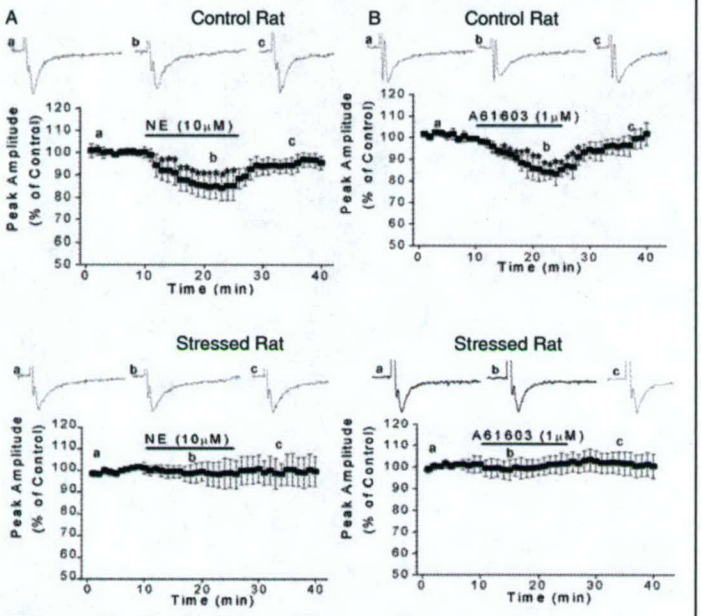

Figure 7. Activation of α_{1A} adrenoceptors reduces BLA field potentials in control rats, but not in stressed rats. (A) Changes in the peak amplitude of BLA field potentials evoked by stimulation of the external capsule, in response to bath application of 10 μM NE, in control (top panel, n = 9) and stressed (bottom panel, n = 10) rats. The medium contains propranolol (10 μM) and yohimbine (20 μM). (B) Similar data to those in (A), except that A61603 is applied in place of the NE. Pooled data from 10 slices (control rats, top panel) and 8 slices (stressed rats, bottom panel). Slice medium same as in (A). Asterisks over error bars denote statistically significant reduction ($p < 0.05$).

Since activation of α_{1A} adrenoceptors facilitates GABAergic transmission, the function of these receptors at the network level could be to dampen neuronal excitability and responsiveness. However, while spontaneous GABAergic activity is dramatically enhanced by activation of α_{1A} adrenoceptors (Fig. 2), evoked GABAergic transmission is suppressed due to presynaptic inhibition of GABA release via $GABA_B$ autoreceptors (Fig. 3). Therefore, under physiological conditions, when $GABA_B$ receptors are not blocked, α_{1A} adrenoceptor activation could enhance amygdala's responsiveness (due to the reduction in evoked GABA release), unless the enhancement of spontaneously released extracellular GABA plays a more decisive role on neuronal excitability. To determine the net effect of α_{1A} adrenoceptor activation on neuronal responsiveness and

excitability in the BLA, and whether this effect is altered by stress, we investigated the effects of NE or A61603 on population field responses, in the absence of $GABA_B$ receptor blockade, in control and stressed rats.

Field potentials in the BLA were evoked by stimulation of the external capsule. These responses consist of one major negative component that corresponds in time course to the EPSP recorded intracellularly from BLA pyramidal cells, and is mediated by AMPA/kainate receptors (Aroniadou-Anderjaska et al., 2001). In control rats, 10 μM NE, in the presence of propranolol (10 μM) and yohimbine (20 μM), produced a significant reduction in the peak amplitude of evoked field potentials (83.8 ± 5.3% of control levels, $n = 14$, $p<0.05$; Fig. 7A). Similarly, bath application of 1 μM A61603 caused a significant reduction in peak amplitude of the field potentials to 83.1 ± 5.2% of control levels ($p<0.05$, $n = 12$; Fig. 7B). In contrast, in stressed rats, neither NE (10 μM) nor A61603 (1 μM) had a significant effect on the amplitude of the field potentials (Fig. 7). These results suggest that the function of α_{1A} adrenoceptors in the BLA is to reduce neuronal excitability/responsiveness and that this function is impaired by stress.

Stress blocks the α_{1A} adrenoceptor-mediated modulation of Long-Term Potentiation.

The memories of traumatic, stressful events are thought to be stored in neocortical areas, with the amygdala playing a major role in modulating the consolidation of these memories. Furthermore, the amygdala itself is thought to be the site of memory formation during stressful experiences that are similar to fear-conditioning. At the cellular level, memory traces are believed to be formed by enduring strengthening of neuronal synapses, within specific neuronal networks. Changes in synaptic strength are expressed as changes in the efficacy of synaptic transmission, such as Long-Term Potentiation (LTP). Both fear-conditioning and other types of stressors are associated with changes in the efficacy of synaptic transmission in the amygdala. Using our stress model, we have found that stress alters the noradrenergic modulation of LTP in the BLA in a manner that facilitates the induction of LTP. Thus, in control rats, theta-burst stimulation (TBS) induces an NMDA receptor-dependent, input-specific LTP of the BLA field potential evoked by stimulation of the external capsule (Fig. 8).

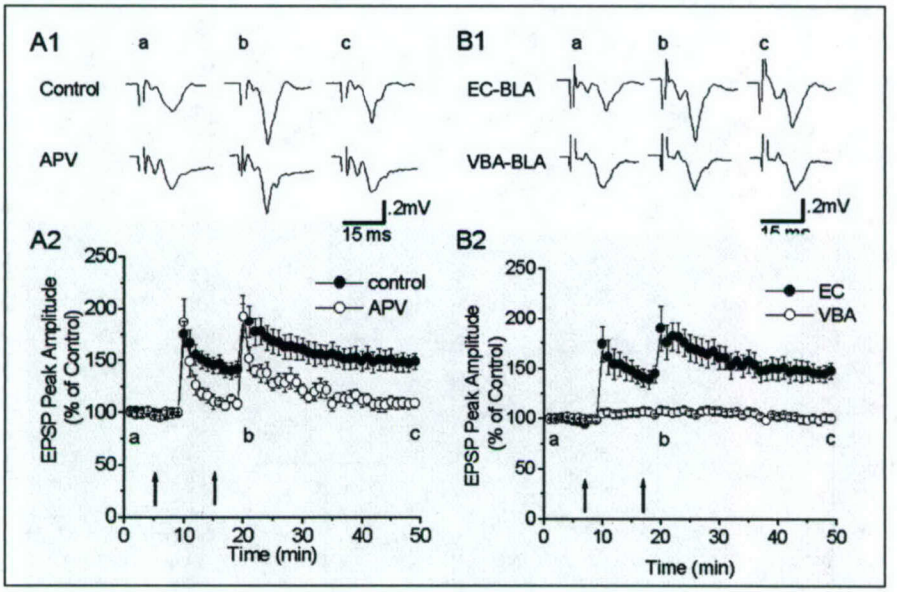

Figure 8. Two theta-burst stimulations (TBS, interval=10 min) of EC induce LTP in the BLA. A: LTP is induced by two TBS of EC (filled circles) and is blocked by NMDA receptor antagonist APV (100 μM, open circles). The peak amplitude of EPSPs 30 min after the second TBS was 149.4±7.9% (n=10) in control and 109.4±4.6% (n=5) for field potentials in the presence of APV. **B**: Two TBS (interval=10 min) of EC induced-LTP (filled circles) is not associated with any change in responsiveness to BA stimulation (open circles, without TBS in VBA-BLA pathway). The peak amplitude of EPSPs 30 min after the second TBS was 148.5±7.9% (n=5) in EC-BLA while in VBA-BLA pathway the EPSPs remained 100.3±2.9% (n=5) of baseline values. The values are expressed as a percentage of the mean of 60 responses at 0.1 Hz before the application of TBS. Each point represents the mean±SEM. TBS was applied at times shown by arrows. Insets show typical traces of EPSPs at the times indicated by the letters **a**, **b** and **c**. Traces are the averages of 6 consecutive responses to the stimulation at 0.1 Hz.

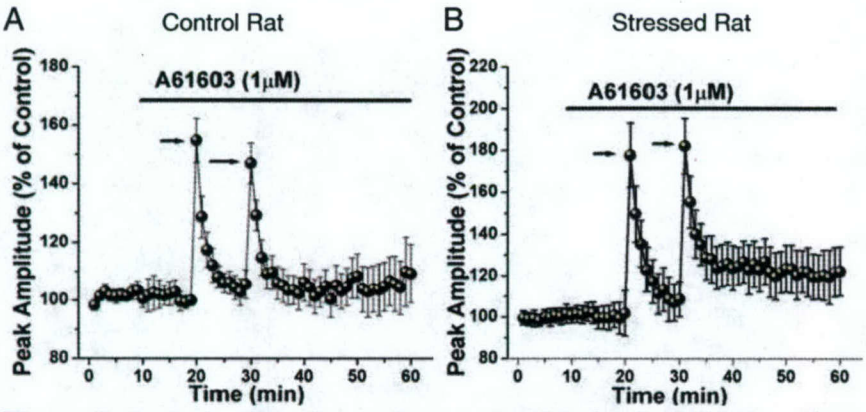

Figure 9. A: Activation of α_{1A} adrenoceptors blocks the induction of LTP. Group data from 12 animals. Error bars are standard errors. **B:** Stress blocks the α_{1A} adrenoceptor-mediated inhibition of LTP. Group data from 14 animals. Error bars are standard errors.

During simultaneous field potential and intracellular recordings, the time course of LTP of the field potential corresponds to that of the EPSP, as we have observed previously (Aroniadou-Anderjaska et al., 2001).

In the presence of the α_{1A} adrenoceptor agonist A61603, TBS does not induce LTP. A61603 reduces evoked field potentials (Fig. 9). LTP is blocked by A61603 whether TBS is applied to the reduced field potentials or after the stimulation intensity is increased to produce a field potential of the same amplitude as the control (in the absence of A61603). In stressed rats, the presence of A61603 does not prevent the induction of LTP (Fig 9). Thus, the stress-induced impairment in the function of α_{1A} adrenoceptors affects the modulation of LTP in the BLA, in a manner that facilitates the induction of LTP. The stress-induced blockade of the α_{1A} adrenoceptor-mediated suppression of LTP may be one of the mechanisms responsible for the over-consolidation of memories associated with stressful events in PTSD patients.

The effects of α_2 adrenoceptor activation on the norepinephrene-mediated modulation of GABAergic synaptic transmission in the basolateral amygdala neurons of control and stressed rats

By means of the whole-cell patch clamp technique, we have examined the hypothesis that the noradrenergic modulation of GABAergic transmission in the BLA is also mediated via activation of α_2 adrenoceptors.

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded from BLA pyramidal neurons at a holding potential of –70 mV, and in the presence of D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM) and prazosin (1 μM). In control rats, the mean frequency of sIPSCs recorded in the soma of BLA pyramidal neurons was 3.6 ± 2.2 Hz. Bath application of bicuculline (10 μM) eliminated sIPSCs, confirming that they were mediated by $GABA_A$ receptors. NE (10 μM) caused a significant decrease of the mean sIPSC frequency that persisted throughout the application of NE and was

completely reversed after removal of the agonist (Fig. 10). These effects of NE were not accompanied by any significant change in the rise time or decay time constant of sIPSCs, and were blocked by the α_2 adrenoceptor antagonist yohimbine (20 μM), confirming that NE was acting via α_2 adrenergic receptors. These results indicate that activation of α_2 adrenoceptors modulates the action potential-dependent release of GABA from BLA interneurons and thus, suggest that these receptors may play an important role in the regulation of the overall excitability of this brain region.

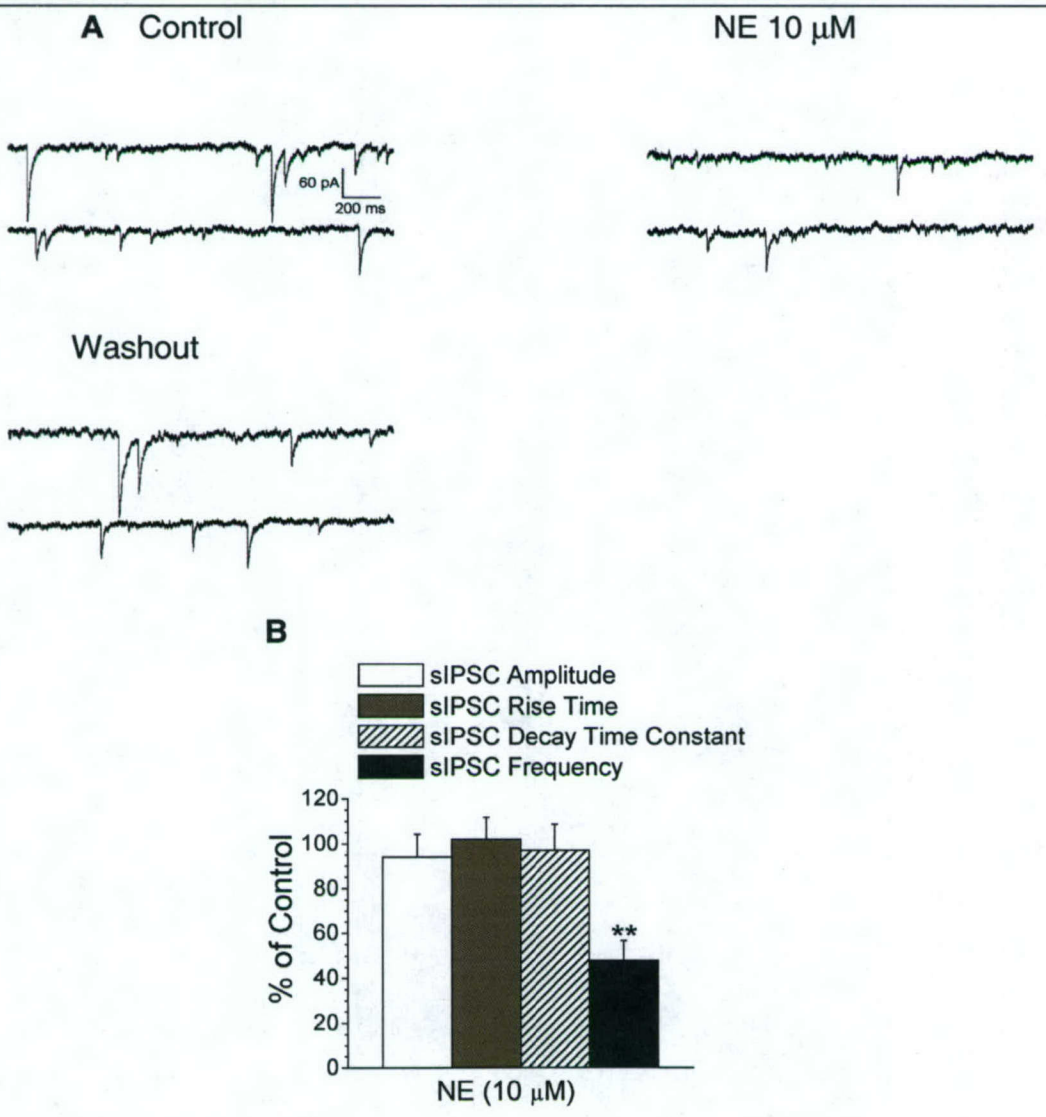

Figure 10. Activation of α_2 adrenoceptors reduces tonic inhibition of BLA pyramidal neurons. (**A**) Top trace: Effects of NE (10 μM) on spontaneous IPSCs (sIPSCs) recorded from a BLA pyramidal cell. Holding potential is -70 mV. The medium contains D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM) and prazosin (1 μM). (**B**) Bottom graph: Pooled data (mean ± SEM) from 8 neurons. Bar graph shows the NE-induced changes in amplitude, frequency and kinetics of sIPSCs. **$p<0.01$.

We have also investigated whether NE is able to modulate evoked inhibitory transmission in the basolateral amygdala via activation of α_2 adrenoceptors. To determine the effects of NE on evoked inhibitory transmission in the BLA, we applied 10 μM NE while recording evoked IPSCs (eIPSCs). In the presence of SCH50911 (20 μM), a specific antagonist of $GABA_B$ receptors, NE significantly reduced the amplitude of evoked IPSCs without affecting the rise time and decay time constant of the evoked IPSCs (Fig. 11A). These effects were mediated via the activation of α_2 adrenoceptors because they were blocked by yohimbine (20 μM, Fig. 11).

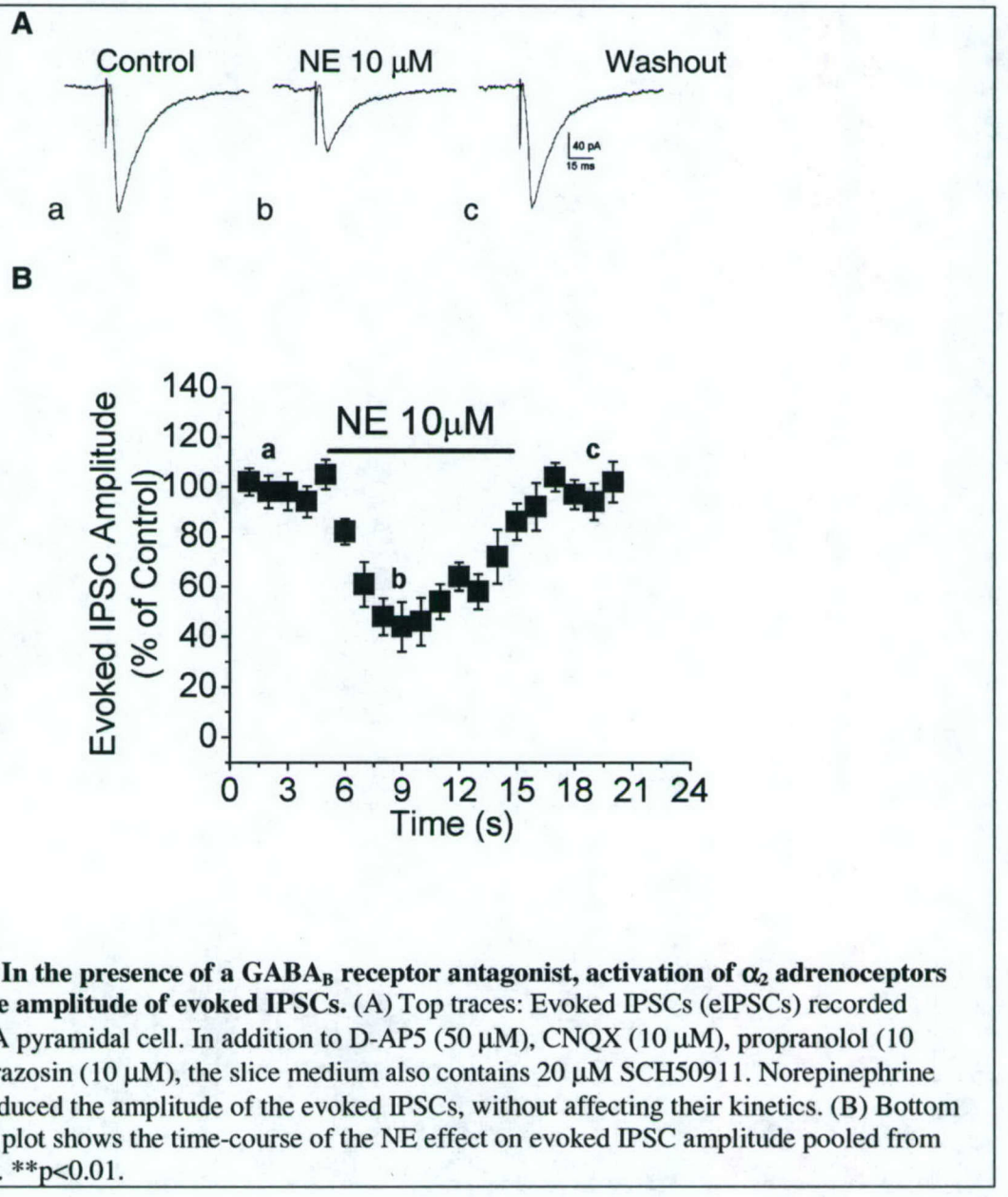

Figure 11. In the presence of a $GABA_B$ receptor antagonist, activation of α_2 adrenoceptors reduces the amplitude of evoked IPSCs. (A) Top traces: Evoked IPSCs (eIPSCs) recorded from a BLA pyramidal cell. In addition to D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM) and prazosin (10 μM), the slice medium also contains 20 μM SCH50911. Norepinephrine (10 μM) reduced the amplitude of the evoked IPSCs, without affecting their kinetics. (B) Bottom graph: The plot shows the time-course of the NE effect on evoked IPSC amplitude pooled from 10 neurons. **$p<0.01$.

The role of β adrenergic receptors on the synaptic function, neuroplasticity and calcium signaling patterns in basolateral amygdala neurons in control and stressed rats in vitro.

In the normal amygdala, basal levels of NE, acting via β adrenoceptors, will contribute to tonic excitation of pyramidal neurons in the BLA by facilitating glutamate release. Enhancement of this facilitation would result in hyperexcitability in amygdala related behaviors. When the normal amygdala is activated in response to an emotionally significant event, triggering the release of NE, activation of β adrenoceptors will facilitate excitatory transmission in amygdala neuronal circuits to enhance emotional arousal. The hyperactivity and hyper-responsiveness of the amygdala associated with certain affective disorders, such as PTSD, has been hypothesized to be due to the loss of proper cortical modulation of the amygdala, and/or due to an intrinsic lower threshold of amygdala response to emotionally significant stimuli. In addition, because the amygdala, and the BLA in particular, plays a central role in the consolidation and reconsolidation of emotional memories and because β adrenoceptors play a key role in learning and memory functions, an up-regulation of β adrenoceptor function in the BLA may be one of the mechanisms underlying the over-consolidation of memories of traumatic events observed in individuals suffering from PTSD. Thus, during stress exposure, excessive release of NE in the amygdala may be responsible for maintaining β adrenoreceptor mediated functions.

β adrenoceptor antagonists as a potential therapy to prevent stress-induced alterations in amygdala physiology and function

Clinical evidence indicates that stress-related affective disorders such as PTSD are associated with changes in amygdala excitability. Thus, one of the diagnostic criteria for PTSD is an exaggerated startle response, which is thought to be mediated by the amygdala. PTSD is also characterized by hyperactivity of the noradrenergic system and a sustained hyperactivation of fear-related neural circuits in the brain. This results in hyperexcitability of the amygdala, which can be responsible for the exaggerated responses to fearful stimuli. In addition, the over-consolidation of memories of traumatic events in PTSD patients may be related to enhanced β adrenoceptor-mediated synaptic potentiation. If this theory is correct, then administration of antagonists of β adrenoceptors to block these receptors before or soon after the occurrence of a traumatic event could have a preventive effect. In support of this thesis, recent clinical trials have demonstrated the effectiveness of propranolol in sexually or physically abused children and combat veterans with PTSD. Thus, application of β adrenoceptor antagonists provides a promising therapeutic strategy to prevent or treat PTSD.

Adrenergic modulation of EPSCs

It has been shown previously that β1 receptor activation enhances evoked excitatory synaptic transmission in the amygdala via enhancing NMDA currents and P-type Ca^{2+} channels. More recently, β1-adrenergic receptors, which activate Mitogen

Activated Protein Kinase, were found to contribute to hippocampal long term potentiation (LTP) induced by 5 Hz stimulation. These results indicate that heightened levels of emotional arousal under stressful conditions appear to facilitate memory consolidation largely mediated by β adrenergic receptor activation. However, a detailed understanding of the cellular mechanisms of β receptor modulation of excitatory synaptic transmission as well as of different forms of synaptic plasticity initiated in the amygdala circuitry remains elusive. Using patch clamp, intracellular and field recordings, we were able to demonstrate that β receptor activation enhances excitatory synaptic transmission and neuronal excitability in the basolateral amygdala circuitry (Figure 12, 13 and 14).

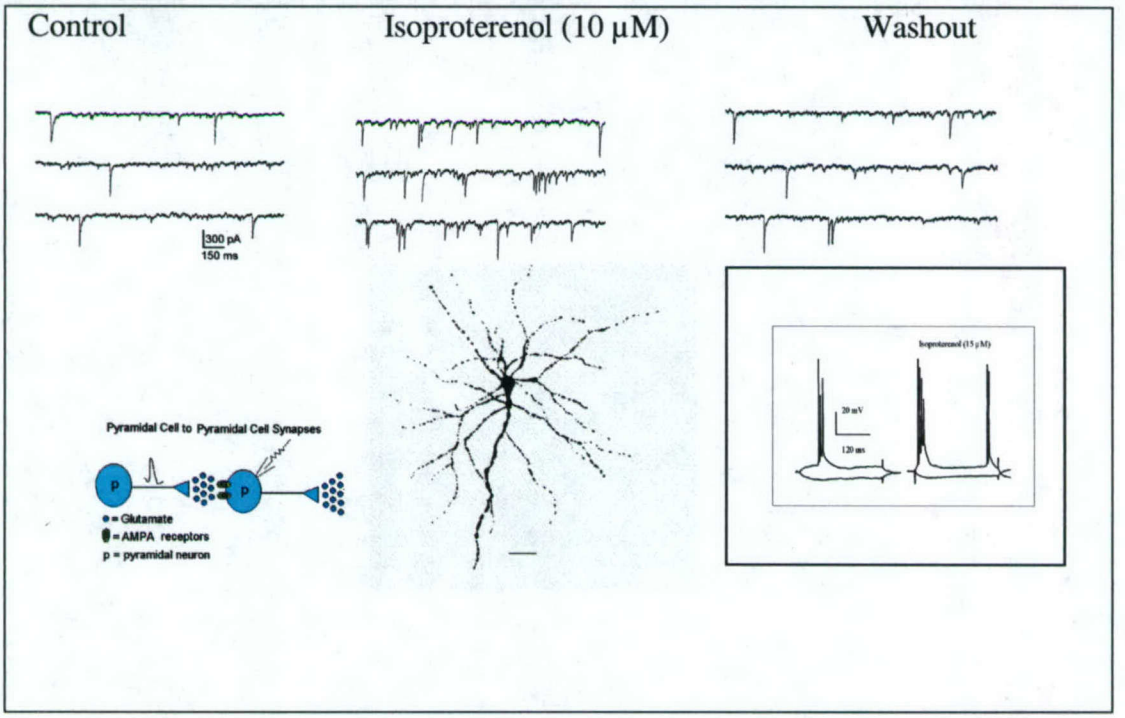

Figure 12. β adrenoceptor agonist, isoproterenol, enhances spontaneous excitatory synaptic currents in the basolateral amygdala. (A) Top left traces: Control spontaneous EPSCs recorded from a BLA pyramidal cell. Top middle traces: Isoproterenol (10 μM) increased the amplitude of the sEPSCs, without affecting their kinetics. Top right trace: washout. Bottom graphs show the cell labeled with Lucifer Yellow and action potentials (insert) evoked by injections of current in the presence of isoproterenol (right) or absence of isoproterenol (left).

β and β2 adrenoceptor agonists, isoproterenol and salmeterol, enhance field excitatory synaptic potential in the basolateral amygdala

As shown in the Figure 13 and 14, the β and β2 adrenoceptor agonists, isoproterenol and salmeterol, both enhance field excitatory synaptic potential in the basolateral amygdala, respectively. These preliminary results indicate that both β1 and β2 receptors are present in the basolateral amygdala complex as isoproterenol, an agonist for both β1 and β2 receptors, enhances field potential from baseline up to

150 % and salmetreol, an agonist specific for β2 receptors, enhances field potential from baseline only up to 125 % on average. The isoproterenol induced facilitation of field potential was abolished in the presence of β adrenoceptor antagonist, propranolol (figure 16). These results are the first detailed demonstration showing that both β1and β2 are participating in the modulation of excitatory synaptic transmission in the amygdala complex. Since activation of β adrenoceptors facilitates excitatory synaptic transmission, the function of these receptors at the network level in the amygdala complex could be to enhance neuronal excitability and responsiveness. Therefore, under stressful conditions, excessive endogenous release of noradrenalin, could act on both β1 and β2 adrenoceptors to enhance amygdala responsiveness.

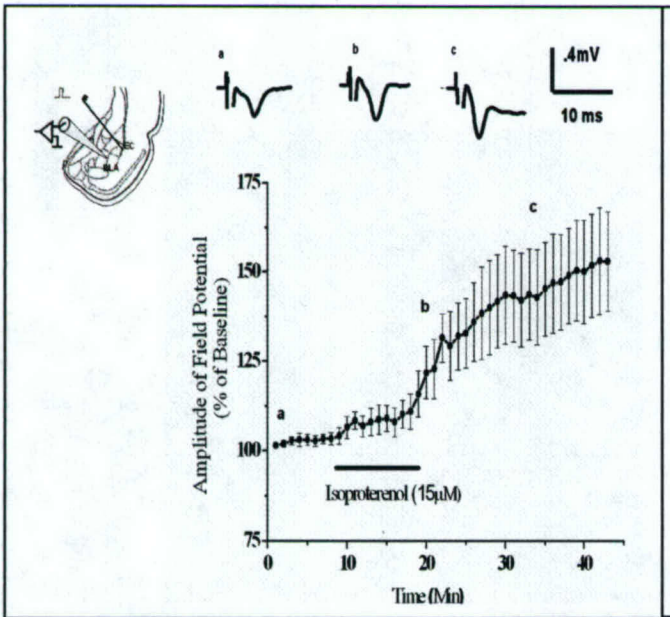

Figure 13. Application of β adrenoceptor agonist, isoproterenol, for ten minutes in the amygdala circuitry is able to enhance the synaptic transmission for a prolonged period of time even after the washout of agonist in the perfusion solution. The field potentials were recorded from basolateral amygdala in slice preparation (n=8)

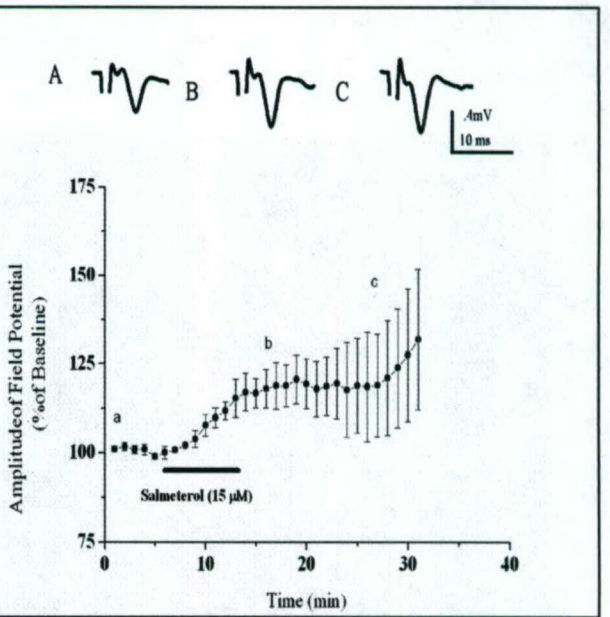

Figure 14. Application of β 2 adrenoceptor agonist, salmeterol, for ten minutes in the amygdala circuitry is able to enhance the synaptic transmission for a prolonged period of time even after the washout of agonist in the perfusion solution. The field potentials were recorded from basolateral amygdala in slice preparation (n=9).

Adrenoceptor β agonist, isoproterenol, enhances intracelluar calcium transience in the basolateral amygdala.

Increased intracellular calcium is considered to be one of the key mechanisms by which memory consolidation is facilitated. Using an intracellular calcium imaging technique, our result (Fig.15) indicates that the β adrenoceptor agonist, isoproterenol, enhances intracelluar calcium signal in basolateral amygdala neurons, thus supporting our hypothesis that β adrenoceptor activation is responsible for facilitating traumatic memory formation and that blocking β

adrenoceptor using a beta receptor antagonist will prevent traumatic memory consolidation.

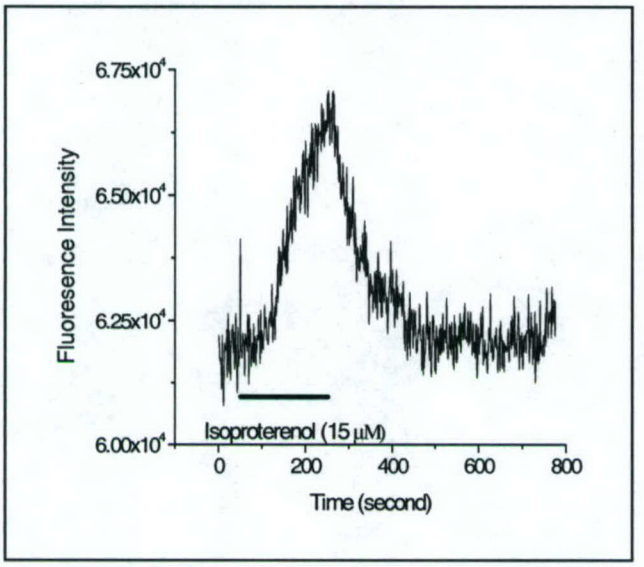

Figure. 15 β adrenoceptor agonist, isoproterenol, enhances the intracellular calcium signaling in the amygdala slice preparation.

Calcium Measurement

Amygdala slices were incubated with ACSF containing 15 μM fura-2 acetoxymethyl ester (Fura-2 AM) and 0.02% Pluronic F127 at 37° C for 30 min and then rinsed in ACSF at room temperature for an additional 15-30 min to remove un-incorporated Fura-2 AM. The slices were transferred to a chamber mounted on an upright Zeiss microscope, then submerged and superfused with ACSF at 2ml/min at room temperature. The microscope was coupled to a DeltaRam monochromator (PTI, Monmouth Junction, NJ) and excitation wavelengths were set to 340 nm and 380 nm. Emitted fluorescence images at 510 nm or higher were captured at a rate of 2 Hz through a 63x Zeiss water immersion objective (N.A. 0.95) with a digital CCD camera (ORCA 100, Hamamatsu, Tokyo, JP) and collected using OpenLab imaging software from Improvision (Lexington, MA). The ratio of emission fluorescence intensity of neuronal somata at 340 nm and 380 nm were calculated with the Improvision software. Peak heights were defined as the difference between the maximum fluorescence ratio value of the smoothed peak and the baseline averaged over 10 s immediately preceding the peak.

Adrenoreceptor β antagonist, Propranolol, blocks effect of isoproterenol on the excitatory synaptic potential in the basolateral amygdala.

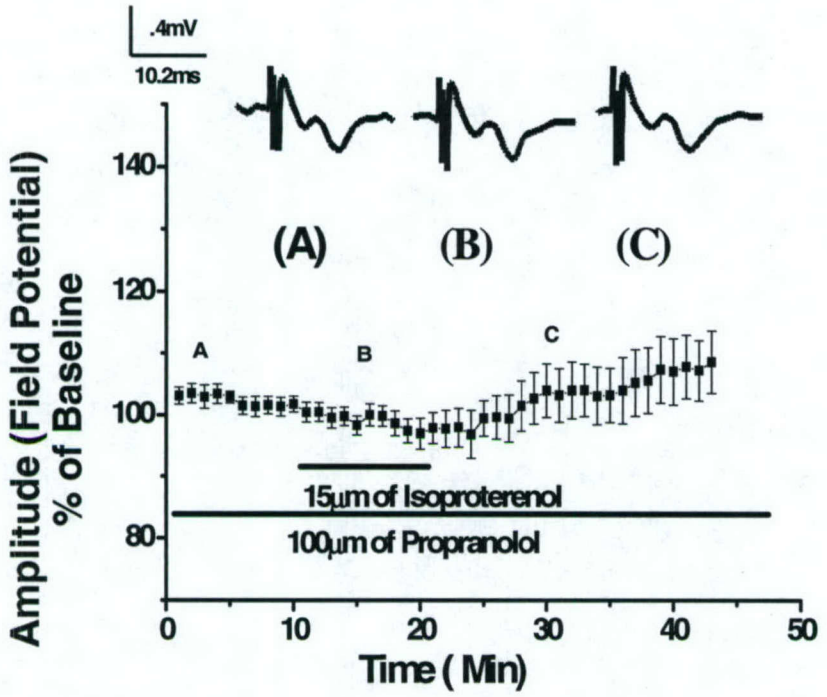

Figure 16: In the presence of β adrenoreceptor antagonist, propranolol, isoproterenol induced potentiation of field potential was abolished (n=8).

β-adrenergic modulation of GluR5 kainate receptor mediated EPSPs

We have investigated the role of isoproterenol, a β adrenoceptor agonist, on BLA GluR5 mediated synaptic transmission by using field and intracellular recordings. NMDA, AMPA, and $GABA_A$ and $GABA_B$ receptor mediated synaptic potentials were blocked by inclusion of 100 μM (-)-2-amino-5-phosphonopentanoic acid, 10 μM bicuculline, 10 μM SCH 50911 and 50 μM GYKI 52466 or GYKI 53655 in the perfusion solution. The effect of β-adrenergic receptor stimulation on intracellular calcium signaling in the BLA neurons was also measured by fluorescence microphotometry with the membrane permeable calcium-specific dye calcium green.

In control rats, external capsule (EC) stimulation evoked GluR5 mediated synaptic responses in BLA neurons that was enhanced by application of the β-adrenergic receptor agonist, isoproterenol (10 μM; 116 12 %) and the synaptic response enhanced by isoproterenol were largely blocked by the AMPA/GluR5 kainate receptor antagonist LY377770 (20 μM; 88 5%) or by the GluR5-selective kainate receptor antagonist LY382884B (100 μM; 86 6%, Fig 17 and 18). In addition, isoproterenol elicited an increase of 10315 2609 (n=16) in the fluorescence intensity equivalent to 21% of the response to 50 mM KCl, indicating an increase in intracellular calcium levels in BLA neurons. These results suggest that enhancement of GluR5 kainate mediated synaptic responses takes place through a β-adrenoceptor elicited intracellular calcium increase in BLA neurons and thus may contribute to the impact of stress on synaptic transmission and synaptic plasticity in the amygdala. We have also examined the effects of isoproterenol (10 μM) on action-potential independent, miniature inhibitory postsynaptic

currents (mIPSCs) recorded from BLA pyramidal neurons in control rats. mIPSCs were recorded at a holding potential of –70 mV, and in the presence of TTX (1 μM), D-AP5 (50 μM), CNQX (10 μM), and prazosin (1 μM). Application of isoproterenol (10 μM) caused a significant increase of the mean sIPSC frequency that persisted throughout the application of the drug and was completely reversed after removal of the agonist.

As initially proposed, we investigated the effects of the β-adrenergic receptor agonist isoproterenol on excitatory synaptic transmission in the basolateral amygdala (BLA). Intracellular recorded excitatory postsynaptic potentials (EPSP) and field excitatory potentials (fEPSP) were evoked by stimulation of the external capsule (EC). GluR5 mediated synaptic responses in BLA neurons were enhanced by application of the β-adrenergic receptor agonist, isoproterenol (10μM; 116 ± 12) and the synaptic response enhanced by isoproterenol was largely blocked by the AMPA/GluR5 kainate receptor antagonist LY377770 (20 μM; 88 ± 5%) or by the GluR5-selective kainate receptor antagonist LY382884B (100μM; 86 ± 6%). These results indicate that the activation of β-adrenergic receptors is able to facilitate GluR5 kainate receptor mediated excitatory synaptic transmission in the neuronal circuitry of the basolateral amygdala. More importantly, this mechanism may be involved in the impact of stress on neuronal communication in the amygdala and could play a role in post-traumatic stress disorder (PTSD).

We have also investigated whether β-adrenergic receptors played a role in the mechanisms underlying low frequency train-induced long-term potentiation in the amygdala. In other words, we tested the hypothesis that during low frequency stimulation these receptors are activated by norepinephrine, which is co-released with glutamate, contributing to the development of enhanced synaptic efficacy. However, bath application of the β-adrenergic receptor antagonists, timolol (20 μM) or propranolol (10 μM) was unable to block the low frequency stimulation-induced synaptic facilitation (Figure 19). When taken together our results suggest that β-adrenergic receptor activation modulates excitatory synaptic pathways in the basolateral amygdala preferentially via facilitation of tonic synaptic neurotransmission rather than by activity-dependent neuronal mechanisms. The involvement of intracellular second messenger systems in the long term potentiation induced by low frequency stimulation will be further examined by using a specific protein kinase A inhibitor in the amygdala slice preparation.

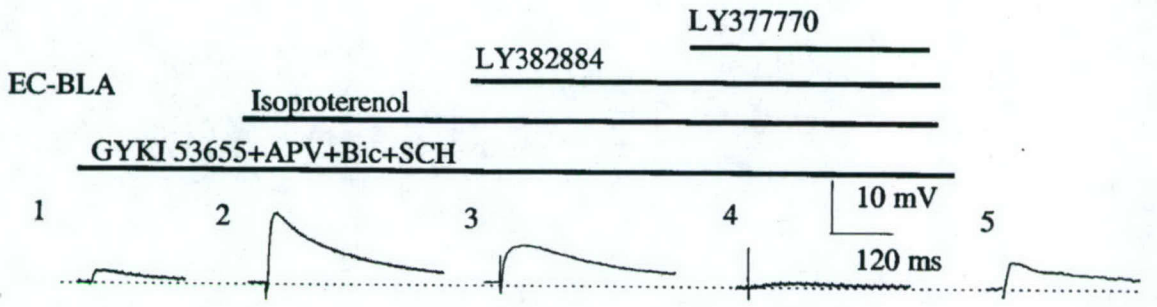

Figure 17. β adrenergic activation enhances GluR5 kainate receptor mediated synaptic potentials in the basolateral amygdala. Shows the average of intracellular recordings of the EC-BLA pathway after addition of GYKI 53655(50 μM) , APV (100 μM), bicuculline (10 μM), and SCH50911 (10 μM, 1), after addition of isoproterenol (2), after addition of LY382884B (3), after the addition of LY377770 (4), and after a washing out period (5).

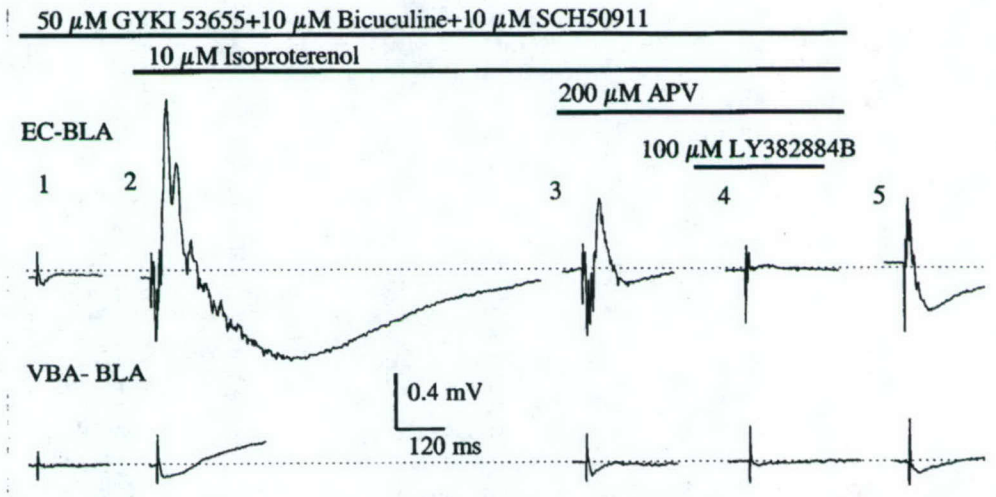

Figure 18. β adrenergic activation enhances GluR5 kainate receptor mediated synaptic potentials in the basolateral amygdala. Shows the average of field recordings of the EC-BLA pathway after addition of GYKI 53655(50 μM), bicuculline (10 μM), and SCH50911 (10 μM, 1), after addition of isoproterenol (2), after addition of APV (3), after the addition of LY382884B (4), and after a washing out period (5).

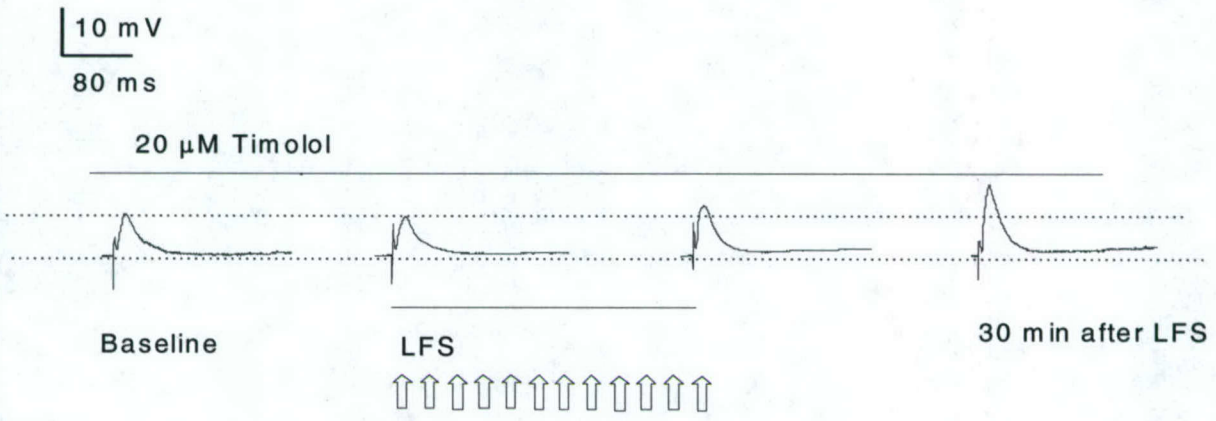

Figure 19. In the presence of timolol, a β adrenergic receptor antagonist, low frequency stimulation remains capable of inducing long term potentiation in the EC and in BLA synaptic transmission.

In summary, we have investigated the effects of stress on the function of β adrenoceptor expression in rat amygdala. The effect of β adrenergic receptor stimulation on intracellular calcium signaling in the BLA neurons of stressed rats was measured by fluorescence microphotometry with the membrane permeable calcium-specific dye fura-2 or calcium green. In contrast to the down-regulation/desensitization of α_1-adrenergic receptors observed after three days of stress, we have observed that the β-adrenergic responses to isoproterenol (these manifest a significant increase in intracellular calcium, as reported in a previous annual report) appear to be up-regulated by stress when tested on the day of or day after the stress. These findings may be very important for understanding what happens immediately after a stressful experience that leads to PTSD.

Stress impairs 5-HT_2 receptor-mediated facilitating effects on synaptic plasticity in the rat basolateral amygdala

Stress-related affective disorders are often associated with changes in emotional learning and memory, implicating possible dysfunction of the neuronal plasticity. Enhanced serotonin release during periods of anxiety and stress has been observed in the amygdala complex (Fernandes et al., 1994). The amygdala expresses high levels of 5-HT_2 receptor protein and mRNAs (Morilak et al., 1994;Pompeiano et al., 1994;Wright et al., 1995). Activation of 5-HT_2 receptors in the amygdala facilitates activity-dependent neuroplasticity in the amygdala circuitry (Wada et al., 1997) and has a profound affect upon anxiety and mood (Hrdina et al., 1993;Kshama et al., 1990;Tokuyama et al., 1993). Therefore, understanding the changes in amygdala neuroplasticity and how its underlying cellular mechanisms are affected by stress is critical in understanding the pathophysiology of stress, and may aid in the development of new therapeutic strategies for the prevention and treatment of stress-related affective disorders.

The amygdala complex is well known for its involvement in mood and emotion (Davis, 1992;Ledoux, 1995), and may participate in the pathogenesis of schizophrenia (Bogerts et al., 1985), depression (Drevets et al., 1992), epilepsy (Boucsein et al., 2001;Pitkanen et al., 1998) and posttraumatic stress disorder (Liberzon et al., 1999;Post et al., 1998;Rauch et al., 2000). In these illnesses, the importance of serotonergic neurotransmission is universally acknowledged.

Excitatory neurotransmission in the basolateral amygdala is mediated by NMDA and AMPA/kainate receptors (Li and Rogawski, 1998;Rainnie et al., 1991). This excitatory neurotransmission exhibits NMDA receptor-dependent and receptor-independent long-term synaptic plasticity (Gean et al., 1993;Li et al., 1998;Li et al., 2001;Maren, 1999) The effect of 5-HT_2 receptor modulation of synaptic plasticity of amygdala circuitry remained to be elucidated. This form of synaptic plasticity may underlie the learning of traumatic memories that characterize fear conditioning, anxiety disorders and posttraumatic stress disorder.

We have examined the impact of stress on the facilitative effect of 5-HT_2-receptor stimulation on synaptic plasticity in the basolateral amygdala, using intracellular and field potential recording techniques. The results demonstrate that stress impairs the facilitative effect of 5-HT_2 receptor activation on theta burst stimulation induced synaptic plasticity in the basolateral amygdala. Such impairment of synaptic plasticity appears to be part of the cellular mechanism underlying emotional learning disorders observed in major depression and post-traumatic stress disorders.

Theta burst stimulations induce short-term potentiation that is not affected by stress. However, the facilitative effect of DOI, a 5-HT_2 receptor agonist, on theta burst stimulation induced long-term potentiation was impaired after stress.

Single theta-burst stimulation (TBS) induced short-term synaptic potentiation in the basolateral amygdala in both control and stressed rats. Figure 20A shows typical traces (averages of 6 responses) of f-EPSPs that were taken from individual experiments at the times indicated by the letters a, b and c. The slope of f-EPSPs 30 min after TBS was 100.9±8.0% (n=6) in control (filled circles) and 107.8±4.4% (n=6) in stress (open circles). In the presence of DOI, a single TBS induced long-term potentiation (LTP) in control rats but failed to induce LTP in stressed rats (Fig. 20 B). The slope of f-EPSPs was 147.6±1.9% (n=6) in control rats (filled circles) and 98.5±6.7% (n=6) in stressed rats (open circles), respectively, 30 min after the onset of TBS. Each point represents the mean ± SEM. Theta-burst stimulation was applied at times shown by arrows. Insets show typical traces of f-EPSPs at the times indicated by the letters a, b and c. Traces are the averages of 6 consecutive responses to the stimulation at 0.1 Hz.

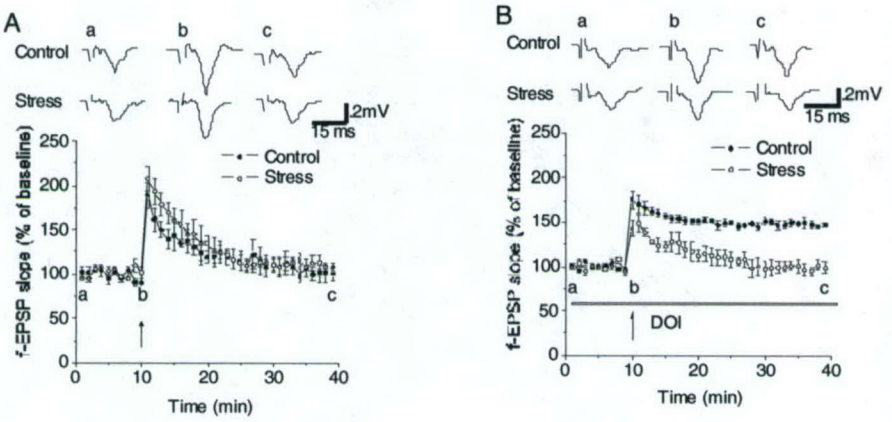

Figure 20. Theta burst stimulation induced short-term potentiation was not affected by stress (A). However, the facilitative effect of DOI, 5-HT2 receptor agonist, on the induction of Long-term potentiation was impaired after stress (B).

The facilitative effect of DOI on the induction of LTP was blocked in the presence of APV, a NMDA receptor antagonist (Figure 21A). Application of APV, an NMDA receptor antagonist, blocked the facilitative effect of DOI on TBS-induced synaptic plasticity in control rats. The cumulative data were presented as the percentage of f-EPSP slopes (in related to the baseline) in the slices treated with APV (50 μM) and DOI (20 μM). The slope of f-EPSPs 30 min after TBS was 109.2±4.3% (n=7) in control rats in the presence of APV and DOI. Figure 20 shows typical traces (averages of 6 responses) of f-EPSPs that were taken from individual experiments at the times indicated by the letters a, b and c. TBS was applied at the times shown by arrows. Bars show the duration of drug application.

The facilitative effect of DOI on NMDA receptor-mediated EPSPs was impaired by stress (Figure 21B). NMDA receptor-mediated EPSPs were enhanced up to 142.9±3.6% (n=7) of the baseline in control rats (filled circles) 15 min after application of DOI. While in the amygdala slices from stressed rats (open circles), the facilitative effect of DOI on NMDA receptor-mediated EPSP was significantly attenuated to

113.0±2.3% (n=7) of the baseline 15 min after the application of DOI. Insets show the typical traces of EPSP at the times indicated by the letters a, b and c.

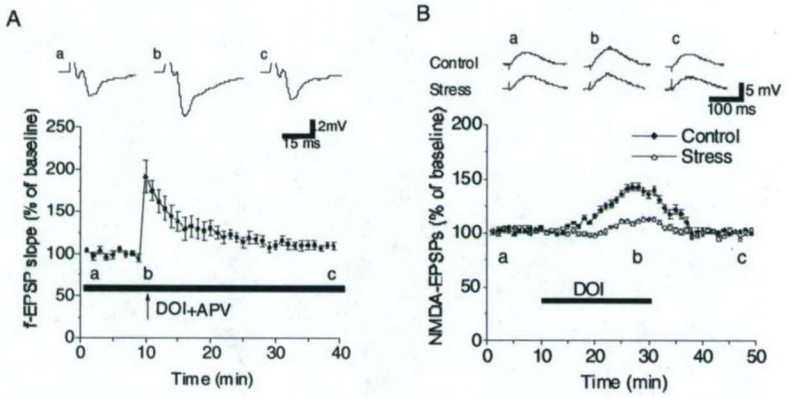

Figure 21. The facilitative effect of DOI on the induction of LTP was blocked in the presence of APV, an NMDA receptor antagonist (A). The enhancement of NMDA-mediated synaptic potential by DOI was significantly attenuated by stress (B).

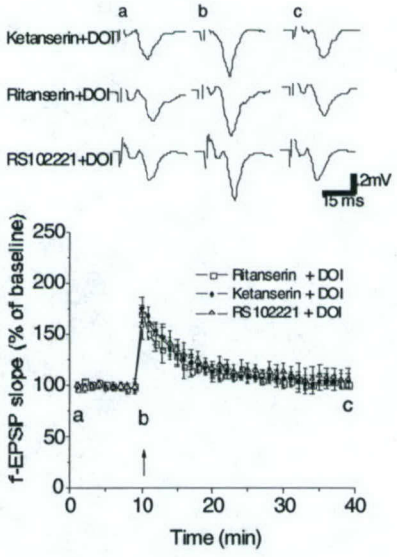

Figure 22. The facilitative effect of DOI on the induction of LTP was abolished in the presence of 5-HT2 receptor antagonists in control rat.

The effects of 5-HT_2 receptor antagonists on the facilitative effect of DOI on TBS-induced synaptic plasticity in control rats are shown in Figure 22. The enhancement of DOI on TBS-induced synaptic potentiation was blocked by the 5-$HT_{2A/2C}$ receptor antagonists ketanserin and ritanserin and by the 5-HT_{2C} receptor antagonist RS 102221 in control rats. The slope of f-EPSPs 30 min after TBS was 106.9±8.7% (n=7) in the presence of ritanserin and DOI, 104.3±7.2% (n=5) in the presence of ketanserin and DOI, 101.4±8.7 (n=5) in the presence of RS 102221 and DOI, respectively. The values are expressed as the percentage of the mean of 60 responses at 0.1 Hz before the application of TBS. Each data point was presented as the mean ± SEM. Theta-burst stimulation was applied at the times indicated by the arrows. The insets show typical traces of f-EPSPs at the times indicated by the letters a, b and c. Each trace is the averages of 6 consecutive responses to the stimulation at 0.1 Hz.

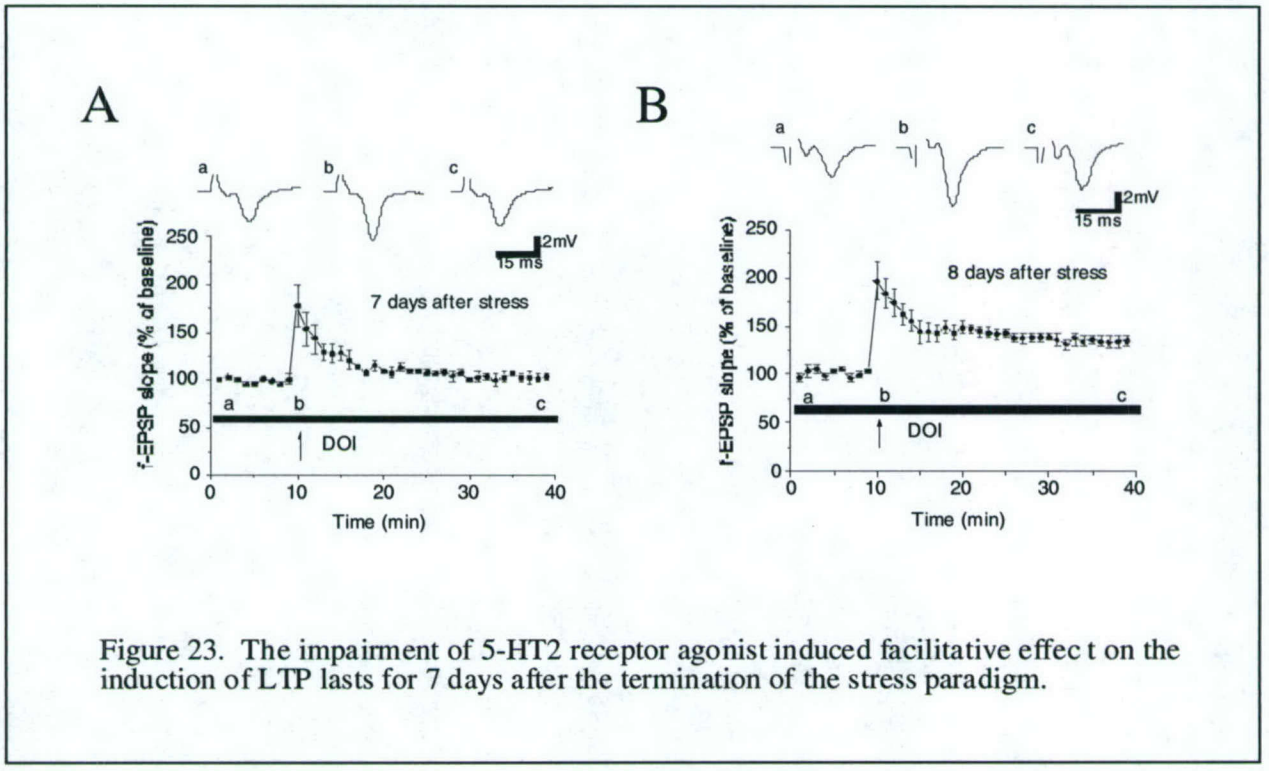

Figure 23. The impairment of 5-HT2 receptor agonist induced facilitative effec t on the induction of LTP lasts for 7 days after the termination of the stress paradigm.

Impairment of 5-HT_2 receptor-mediated facilitative effects on TBS-induced LTP lasts for seven days after stress.

The percentage of f-EPSP slopes was measured in relation to baseline in stressed rat BLA seven days after stress (n=5). DOI remains incapable of facilitating TBS-induced synaptic potentiation seven days after stress. The slopes of f-EPSP 30 min after TBS were 103.5±3.5% (n=5) of the baseline (Figure 23A). The typical traces (averages of 6 responses) of f-EPSPs were taken from individual experiments at the times indicated by a, b and c. TBS was applied at the times indicated by arrows. 23B, The percentage of f-EPSP slopes is presented in relation to baseline in stressed rat BLA eight days after stress (n=7). DOI is again capable of facilitating the effect on TBS-induced synaptic plasticity eight days after stress. The slopes of f-EPSP 30 min after TBS were 134.7±5.3% (n=9) of

the baseline. The typical traces (averages of 6 responses) of f-EPSP were taken from individual experiments at the times indicated by a, b and c. The TBS was applied at the times indicated by arrows.

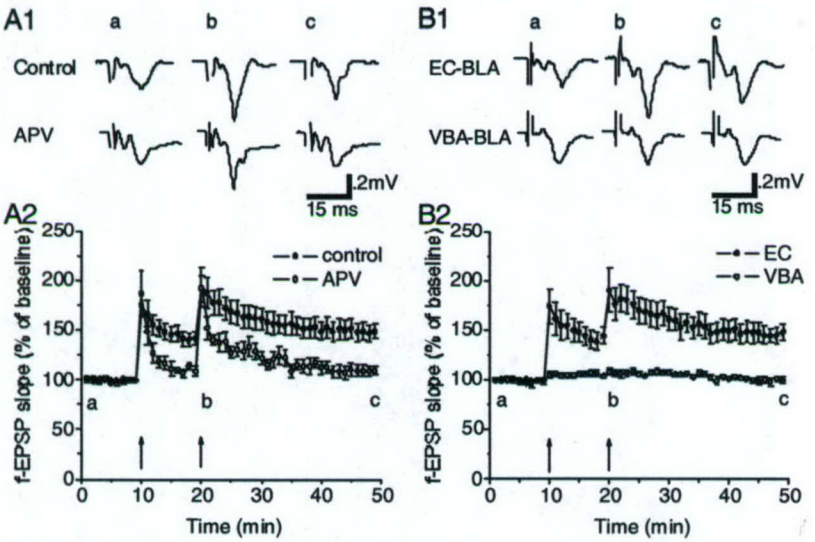

Figure 24. Two theta burst stimulations induce an NMDA receptor-dependent long -term potentiation (A1 and A2), which characterizes a pathway specific synaptic plasticity (B1 and B2).

Two TBS-induced (interval of 10 minutes) LTP is NMDA-dependent and input-pathway specific in the BLA.

LTP was induced by two TBS of EC and blocked by the NMDA receptor antagonist APV. Figure 24 A1 shows typical traces (averages of 6 responses) of f-EPSPs at the times indicated by the letters a, b and c in A2. A2, Mean ± SEM percentage of f-EPSP slope (in relation to baseline) in the slices treated with ACSF (black circles, n=10) or 50 μM APV (white circles, n=5). B, Two TBS of EC induced LTP in BLA that was not associated with any change in responsiveness to ventral basal amygdala (VBA) stimulation (no TBS in VBA-BLA pathway). B1, Typical traces (averages of 6 responses) of f-EPSPs at the times indicated by the letters a, b and c in B2. B2, Mean ± SEM percentage of f-EPSP slope (in relation to baseline) in EC-BLA (black circles) or in VBA-BLA (white circles). TBS was applied at the times shown by arrows.

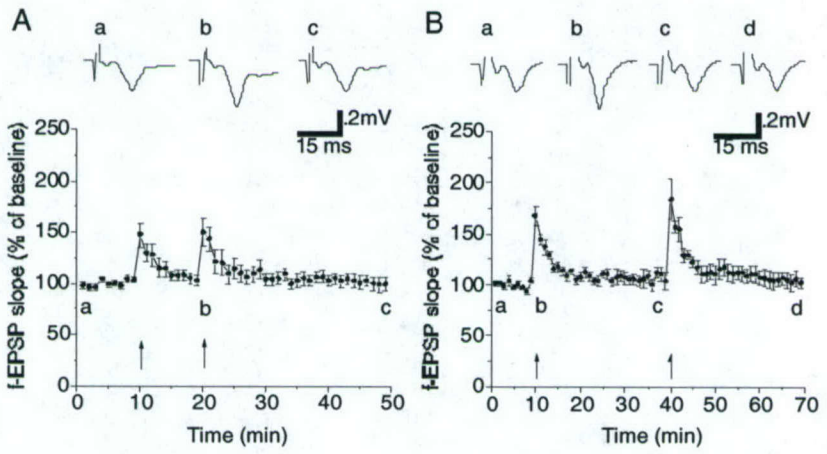

Figure 25. Acute one day stress impairs two theta burst stimulations induced long-term potentiation in the basolateral amygdala immediately after the termination of stress paradigm.

Figure 25 shows that the TBS-induced LTP was impaired after acute (one day) stress. A, Mean ± SEM percentage of f-EPSP slope was presented in relation to baseline in stressed rat BLA (n=7). The interval of two TBS was 10 min. Typical traces (averages of 6 responses) of f-EPSPs were taken from individual experiments at the times indicated by the letters a, b and c. TBS was applied at the times shown by arrows. B, Mean ± SEM percentage of f-EPSP slope was presented in relation to baseline in stressed rat BLA (n=4). The interval of two TBS was 30 min. Typical traces (averages of 6 responses) of f-EPSPs were taken from individual experiments at the times indicated by the letters a, b and c. TBS was applied at the times shown by arrows.

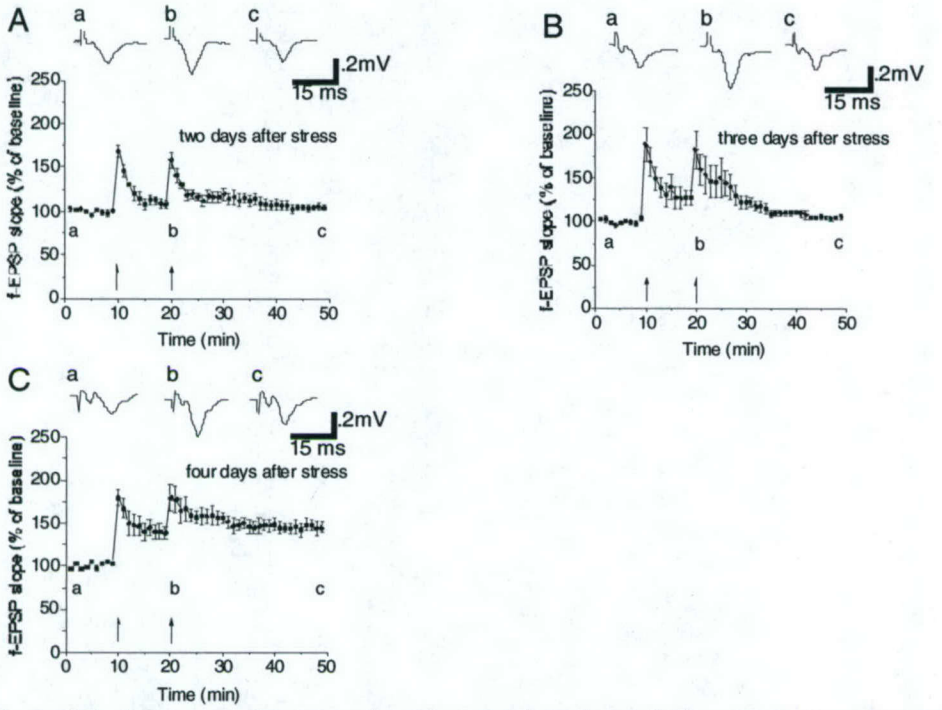

Figure 26. Acute one day stress impairs two theta burst-induced long-term potentiation in the amygdala and the effect of such impairment lasts for three days.

Acute (one day) stress impairs two-theta burst-induced long-term potentiation in the amygdala and the effect of such impairment lasts for three days after termination of the stress paradigm.

In Figure 26A, the Mean ±SEM percentage of f-EPSP slope is presented in relation to baseline in stressed rat BLA two days after stress (n=5). The interval of two TBS was 10 min. Typical traces (averages of 6 responses) of f-EPSPs were taken from individual experiments at the times indicated by the letters a, b and c. TBS was applied at the times shown by arrows. Two TBS, at an interval of 10 min and at an interval of 30 min, failed to induce LTP. The slopes of f-EPSPs 30 min after the second TBS remained 100.3±6.9% (n=7) at an interval of 10 min and 102.9±5.3% (n=4) at an interval of 30 min of the baseline, respectively. B, Mean ± SEM percentage of f-EPSP slope is presented in relation to baseline in stressed rat BLA three days after termination of stress (n=7). TBS-induced LTP was impaired by three-day stress. The slope of f-EPSPs was 106.5±4.0% (n=5) of the baseline 30 min after the second TBS. The interval between the two TBS was 10 min. Typical traces (averages of 6 responses) of f-EPSPs were taken from individual experiments at the times indicated by the letters a, b and c. TBS was applied at the times shown by arrows. C, Mean ± SEM percentage of f-EPSP slope was presented in relation to baseline in stressed rat BLA four days after stress (n=7). Two TBS failed to induce LTP until four days after stress. The slope of f-EPSPs 30 min after TBS was 145.2±6.2% (n=7) of the initial baseline values. The interval between two TBS was 10

min. Typical traces (averages of 6 responses) of f-EPSPs were taken from individual experiments at the times indicated by the letters a, b and c. TBS was applied at the times shown by arrows.

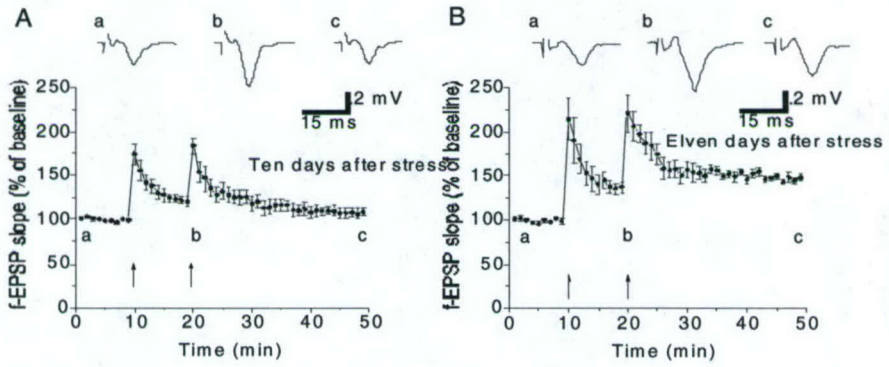

Figure 27, Three days stress impairs two TBS-induced LTP 10 days after the termination stress paradigm(A). The two TBS-induced LTP resumed 11 days after the termination of stress (B).

Stress impairs two theta burst stimulations induced long-term potentiation in the Rat Basolateral Amygdala *in vitro*

We have examined the impact of single and repeat stress on theta burst stimulation induced enduring synaptic potentiation in the basolateral amygdala in vitro, and demonstrated that two theta burst stimulations (applied at 10 minute intervals) of the external capsule (EC) induced a persistent enhancement in the slopes of homosynaptic potentiation in the intercellular recorded single neuron and field potentials recorded in basolateral amygdala circuitry. Single theta burst stimulation induces a short-term synaptic potentiation lasting approximately 10 minutes after the termination of stimulation. The enhancement of two theta burst stimulation induced synaptic potentiation was maintained for more than 30 min following termination of the stimulus train. Two TBS-induced enduring synaptic facilitation was blocked by the N-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonovalerate (APV; 100 μM). The acute one day stress paradigm impairs the induction of two TBS-induced long term potentiation with no significant effect on a single TBS induced short-term potentiation. The impairment of two TBS induced LTP by one day stress lasted for three days and the two TBS induced LTP was resumed on the fourth day after the termination of stress.

Repeat of the three day stress paradigm also impairs the induction of two TBS-induced long-term potentiation with no significant effect on a single TBS induced short-term potentiation. The impairment of two TBS induced LTP by three days stress lasted for ten days and the two TBS induced LTP resumed on the eleventh day after the termination of stress. (Figure 27). The slope of f-EPSPs 30 min after TBS was 148.2±8.9% (n=4, Figure 27B) of the initial baseline values. The result suggests that the recovery of the ability to induce LTP with TBS is a time-dependent recovery from the stress.

The present findings provide insight into possible mechanisms underlying the impact of stress on emotional learning and memory. These results indicate that stressful stimuli have mild impact on the induction of short-term synaptic plasticity, but prolonged effects on the induction of long-term synaptic plasticity in the basolateral amygdala. The time course of resilience from such stressful stimuli is correlated with the intensity of the stressors.

Alterations of alpha 2 adrenergic and serotonergic 5-HT$_2$ receptor functions in the rat amygdala circuitry after traumatic stress

1. Alpha 2 adrenoceptor mediated inhibition of excitatory synaptic transmission in the basolateral amygdala

The previous studies have shown that norepinephrine exerts strong inhibitory effects on synaptic transmission in amygdala circuitry, which appears to be primarily mediated by alpha 2 adrenceptors. Using field potential recording, we confirmed that norepinephrine could dose-dependently inhibit excitatory synaptic transmission in the BLA (Figure 28A). Blockade of alpha 2 adrenoceptors using yohimbine could prevent norepinephrine-induced suppression of field potential (figure 28B), while clonidine, an alpha2 receptor agonist, mimicked the norepinephrine induced inhibitory effect (figure 28C). These observations indicate that alpha 2 adrenoceptors are localized in the glutaminergic terminals and induce suppression of excitatory synaptic transmission.

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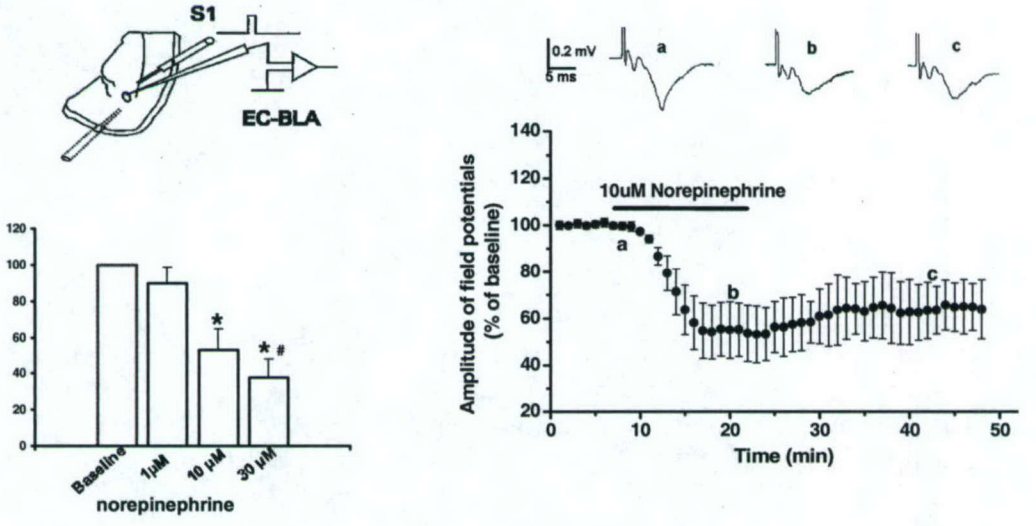

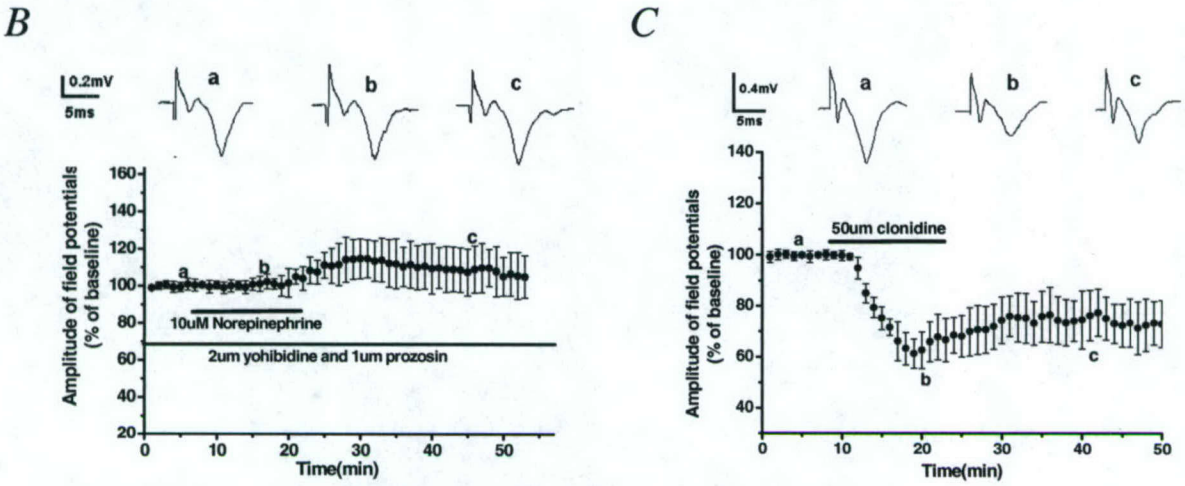

Figure 28. The alpha2 adrenoceptor mediates norepinephrine-induced suppression of field potentials in the BLA. **A**. Norepinephrine dose-dependently suppresses field potentials recorded in the BLA (inset shows the recording and stimulating sites in the BLA slices). **B**. 2μM yohimbine, the alpha2 receptor antagonist, blocks norepinephrine-induced suppression. **C**. Clonidine, the alpha2 receptor agonist, mimics norepinephrine to induce the inhibitory effect on field potentials.

2. Delayed impairment of alpha2 adrenoceptor-mediated inhibition of synaptic transmission by traumatic stress.

In order to investigate whether alpha2 adrenoceptor-mediated inhibition of synaptic transmission is altered after traumatic stress, we assigned randomly the animals to two groups, one control without stress and one receiving restraint/tail shock for three days. Amygdala slices were prepared from these two group rat brains. In the slices from control rats, 50μM clonidine suppressed the field potentials to 66.3±4.5% of control levels (Figure 29A). In the amygdala slices from stressed rats immediately after the stress, clonidine at the same concentration suppresses the field potentials to 56.4 ±6.1% of the control levels (Figure 29B). This result indicates that stress appears to have no significant effect on alpha 2 receptor mediated function in the amygdala immediately after stress paradigm.

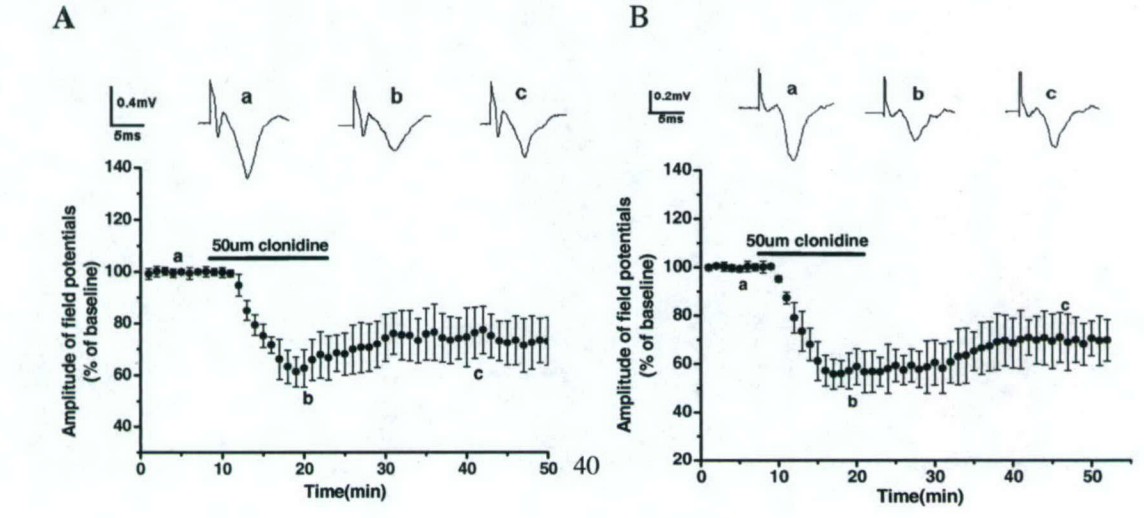

Figure 29. The effect of 50μM clonidine on field potentials in control and stressed rats (0 days or 1 days post-stress). (A) Changes in the peak amplitude of BLA field potentials evoked by stimulation of the external capsule, in response to bath application of 50 μM clonidine, in control rats(n = 10). (B) Changes in the peak amplitude of BLA field potentials evoked by stimulation of the external capsule, in response to bath application of 50 μM clonidine in stressed slices (n = 11)

The syndromes of PTSD are normally observed three months after traumatic experiences in humans. Thus, we determine the effect of alpha2 receptor agonist seven days after the termination of stress. As indicated in figure 30 alpha2 receptor mediated inhibition of synaptic transmission was attenuated seven days after termination of stress. In the slices from these rats, 50μM clonidine suppressed the field potentials to 77.±7.6% of the baseline levels (Figure 30A,n=20), which was significantly different from the control (62.2± 5.7% of baseline, n=7) (figure 30B). These results indicate that alpha2 receptor-mediated synaptic function was severely impaired in stressed animals seven days after stress.

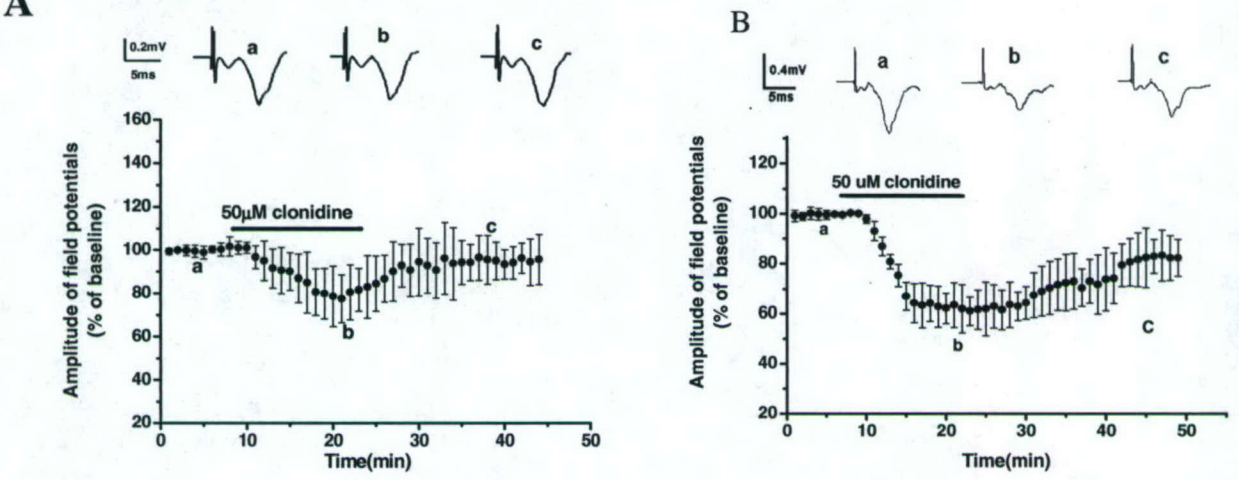

Figure 30. The effect of 50μM clonidine on field potentials in control and stressed rats (7 days to 10 days post-stress). (**A**) Changes in the peak amplitude of BLA field potentials evoked by stimulation of the external capsule, in response to bath application of 50 μM clonidine, in stressed rats (n = 20). (**B**) Changes in the peak amplitude of BLA field potentials evoked by stimulation of the external capsule, in response to bath application of 50 μM clonidine in control (n = 7)

3. 5-HT facilitates GABA release in the lateral nucleus of the amygdala.

Coronal amygdala slices from male Sprague Dawley rats weighing 40 to 60 gm was prepared as described above. For whole-cell recordings slices are transferred to a submersion-type recording chamber where they are continuously perfused with oxygenated ACSF at a rate of 4 ml/min. All experiments were carried out at 32°C. Tight-seal (>1 GΩ) whole cell recordings are obtained from the cell body of neurons in the BLA region. Neurons are visualized with an upright microscope (Nikon Eclipse E600fn) using Nomarski-type differential interference optics through a 60X water immersion objective. During whole-cell recordings, neurons are filled passively with 0.4% Lucifer yellow (Molecular Probes, Eugene, Oregon) for post hoc morphological identification described by our laboratory. Neurons are voltage clamped using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA). Inhibitory postsynaptic currents (IPSCs) are pharmacologically isolated and recorded at a holding potential of –70 mV. Synaptic responses are evoked with sharpened tungsten bipolar stimulating electrodes (2 μm diameter, World Precision Instruments, Sarasota, Florida) placed in the BLA, 50-100 μm from the recording electrode. Stimulation is applied, at 0.1 Hz, using a photoelectric stimulus isolation unit having a constant current output (PSIU6, Grass Instrument CO., W. Warwick, RI). Access resistance (8-6 MΩ) is regularly monitored during recordings, and cells are rejected if it changed by more than 15% during the experiment. The signals are filtered at 2 kHz, digitized (Digidata 1322A,Axon Instruments, Inc.), and stored on a computer using the pCLAMP8 software (Axon Instruments, Inc.). The peak amplitude, 10-90% rise time, and decay time constant of IPSCs are analyzed off-line using pCLAMP8 software (Axon Instruments) and the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). Miniature IPSCs are analyzed off-line using the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ), and detected by manually setting the threshold for each mIPSCs after visual inspection.

In whole-cell patch clamp, spontaneous IPSCs were recorded at a holding potential of –70 mV, and in the presence of D-AP5 (50 μM), CNQX (10 μM), to block NMDA, AMPA/kainate receptors, respectively. As shown in the figure 31, administration of serotonin dramatically increase the frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from BLA pyramidal neurons.

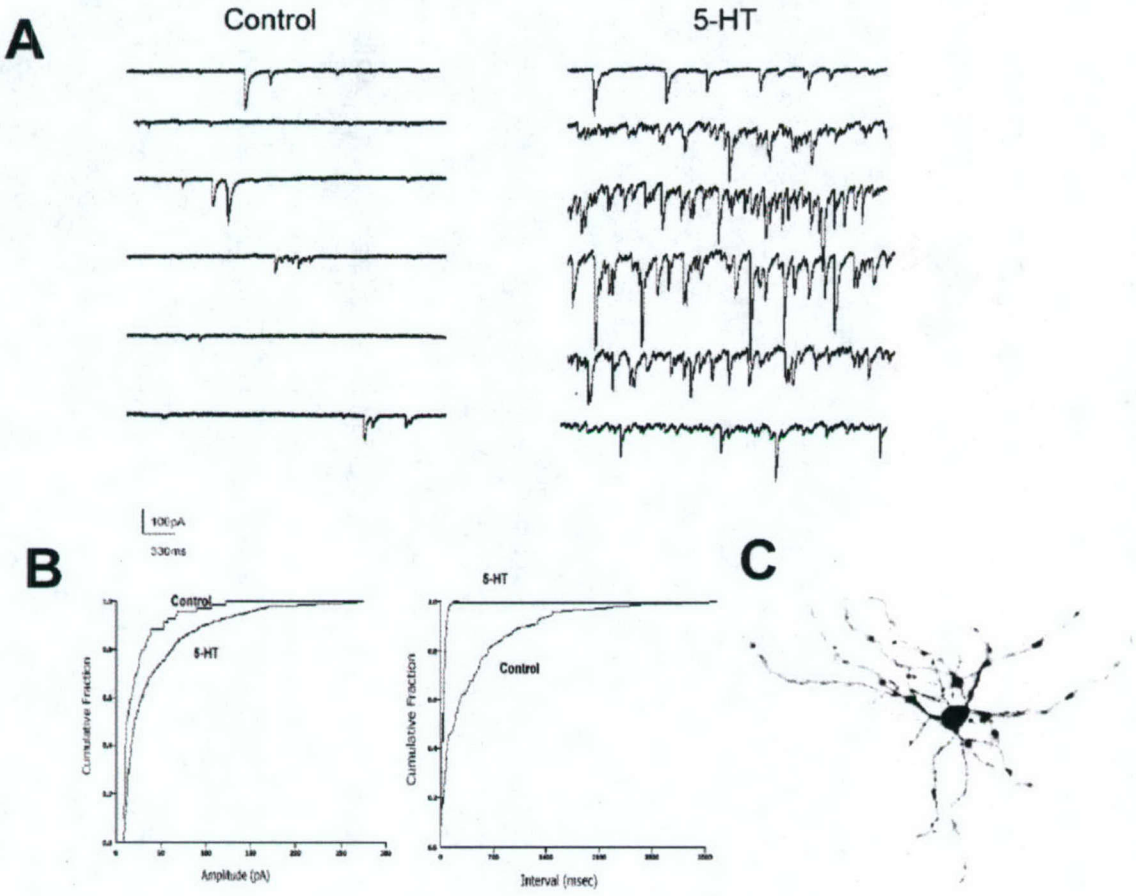

Figure 31. Effect of 5-HT on spontaneous GABAergic IPSCs in the lateral nucleus of the amygdala. A, in the presence of 5-HT3 receptor antagonist Y-25130 (1μM) and 5-HT1 receptor antagonist cyanopindolol (10μM), administration of 5-HT(50μM) dramatically enhances the frequency of sIPSCs and shifts their amplitude distribution curve toward larger sizes (n=10). Holding potential:-70mV. Holding current: -20pA. B, Cumulative amplitude and frequency distributions for the same experiment. 5-HT elicits a significant shift to the right in the cumulative amplitude distribution ($P<0.05$, K-S test). It also elicits a significant shift to the left in the cumulative distribution of inter-sIPSC intervals corresponding to the increase in sIPSC frequency ($P<0.05$, K-S test). C, Photomicrograph of pyramidal cell showing the typical morphology of the recorded neurons. The cell has been labeled with Lucifer Yellow. Scale bar, 40 μm.

4. 5-HT_2 receptors may underlie the facilitatory effect of 5-HT on sIPSCs

Previous studies suggest that 5-HT_2 receptors are responsible for 5-HT-induced GABA release in the BLA. As shown by figure 32, 5-HT_2 receptor agonist alpha-methyl–5-HT could mimic 5-HT to facilitate the sIPSCs. To further determine the 5-HT_2 receptor

subtypes responsible for such effect, a selective 5-HT_{2C} agonist and selective 5-HT_{2A} antagonists were used in the subsequent studies.

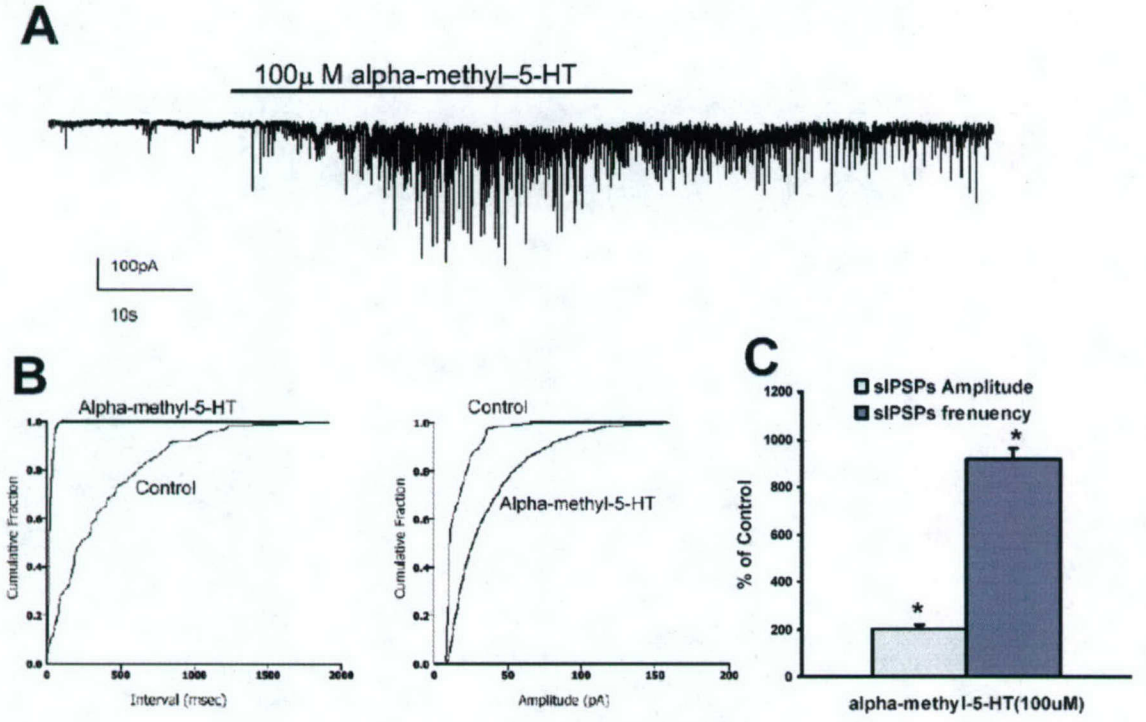

Figure 32. 5-HT_2 receptor agonist, alpha-methyl–5-HT mimics 5-HT mediated effect and facilitates the sIPSCs. A, alpha-methyl-5-HT dramatically increases frequency and amplitude of sIPSCs. Holding potential: -70mV. B, Cumulative amplitude and frequency distributions from the same experiment were plotted in figure 35 B. alpha-methyl–5-HT elicits a significant shift of curve to the right in the cumulative amplitude distribution ($P<0.05$, K-S test). It also elicits a significant shift to the left in the cumulative distribution curve of inter-sIPSC intervals corresponding to the increase in sIPSC frequency ($P<0.05$, K-S test). C, Group data of amplitude and frequency of sIPSCs during administration of alpha-methyl–5-HT compared to the control (n=9, *$P<0.01$)

5. To determine whether 5-HT-induced facilitation of GABA release in the BLA is severely impaired by the traumatic stress.

Male Sprague Dawley rats weighting 40 to 60 gm were randomly assigned to the control group and stress group. Control rats are kept undisturbed, while stress group were given the stress protocol as described in the proposal. After termination of the stress, both control and stressed rats are sacrificed and slices are prepared for further experiments.

We first examine the protein level of 5-HT_{2A} receptor in the amygdala and hippocampus using Western Blot analysis. Traumatic stress significantly decreases 5-HT_{2A} receptor protein level specifically in the amygdala (n=6), without affecting this receptor expression in the hippocampus (figure 33).

These results confirm our previous observation that 5-HT_2 receptor mediated facilitation action on the induction of LTP in the amygdala is greatly attenuated after stress.

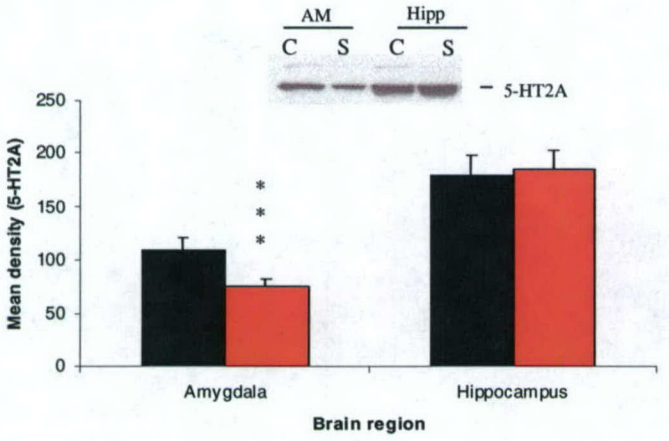

Figure 33. The effect of traumatic stress on 5-HT_{2A} protein levels in the hippocampus and amygdala. Protein levels were determined by Western blot analysis. 100 μg of total protein from the hippocampus and amygdala was loaded. *** $P < 0.001$.

KEY RESEARCH ACCOMPLISHMENTS

The ultimate goal of this research is to acquire and provide the knowledge that is necessary for the development of novel and effective pharmacological means that will prevent or treat stress-related affective disorders, such as post-traumatic stress disorder (PTSD). The amygdala has been the focus of this study because previous studies have suggested a central role for the amygdala in stress-related emotional illnesses, both in modulating the consolidation of emotional memory and in acting as a storage site for emotional memories. Teamwork on the part of all members of the He Li's laboratory has allowed us to accomplish and even surpass the goals of this project as recorded in the Statement of Work. A brief summary follows:

(1) We have established an animal model of PTSD. Using an inescapable tail-shock paradigm in rats, we have demonstrated the ability of stress to induce elevated basal corticosterone levels, weight loss and exaggerated startle response, all of which have been observed in PTSD patients.

(2) We have completed the work proposed in each of the five Specific Aims listed in the statement of work. This includes the electrophysiological measurements made on neurons in the basolateral amygdala (BLA) (Specific Aim 1) and, with the use of appropriate agonists and antagonists, the characterization of noradrenergic receptors (α1, α2, and β) and serotonergic receptor ($5HT_2$) roles on the modulation of neuroplasticity and calcium signaling patterns in the basolateral amygdala (Specific Aims 2-5). In one of the most significant findings resulting from this work, we have observed the loss of alpha 1A adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala following exposure to stress. These findings provide important insights into possible mechanisms underlying the hyperexcitability of the amygdala in certain stress-related disorders such as PTSD,

(3) We have examined the impact of stress on the facilitative effect of 5-HT_2-receptor stimulation on synaptic plasticity in the basolateral amygdala, using intracellular and field potential recording techniques. The results demonstrate that stress impairs the facilitative effect of 5-HT_2 receptor activation on the theta burst stimulation induced synaptic plasticity in the basolateral amygdala. Such impairment of synaptic plasticity appears to be part of the cellular mechanism underlying emotional learning disorders observed in major depression and post-traumatic stress disorders. One paper has been published in *Neuroscience* and one is in preparation for publication.

(4) We have also revealed that prolonged low-frequency stimulation of excitatory afferents to basolateral amygdala neurons results in enduring enhancement of excitatory synaptic responses. The induction of this form of heterosynaptic plasticity is eliminated by selective antagonists of glutamate 5 kainate receptor (GluR5). Such heterosynaptic spread of synaptic facilitation could account for

adaptive and pathological expansion in the set of critical internal and external stimuli that trigger amygdala-dependent emotional learning and memory. In addition, a dose-dependent bidirectional modulation of γ-amino butyric acid (GABA) release is found to be mediated by presynaptic GluR5 kainate receptors in the BLA.

(5). We have examined the impact of single and repeat stress on theta burst stimulation induced enduring synaptic potentiation in the basolateral amygdala *in vitro*, and demonstrated that two theta burst stimulations (applied at 10 minute intervals) of the external capsule (EC) induced a persistent enhancement in the slopes of synaptic potentiation in the intercellular recorded single neuron and field potentials recorded in basolateral amygdala circuitry. We have demonstrated that stressful stimuli (both one day and three days restrain and tail shock stress) have prolong effects on the induction of long-term synaptic plasticity but have no significant impact on the induction of short-term synaptic plasticity in the basolateral amygdala. The acute one day stress paradigm (extended studies from initial proposal) impairs the induction of two TBS-induced long term potentiation. The impairment of two TBS induced LTP by one day stress lasted for three days and the two TBS induced LTP was resumed on the fourth day after the termination of stress. Repeat of the three day stress paradigm impairs the induction of two TBS-induced long-term potentiation with no significant effect on a single TBS induced short-term potentiation. The impairment of two TBS induced LTP by three days of stress lasted for ten days and the two TBS induced LTP resumed on the eleventh day after the termination of stress. The time course of resilience for the induction of long-term potentiation from such stressful stimuli is correlated with the intensity and duration of the stressors.

The identification of these multiple cellular mechanisms affected by stress in the amygdala will provide important detailed information for developing pharmacological interventions to regulate circuit excitability in the amygdala and to ameliorate amygdala related mental disorders such as fear, anxiety, depression and PTSD.

Publications and reports resulting from this project are summarized as follows:

1. Nine papers, including an invited review article, reporting the findings of this project have been published in peer-reviewed journals including ***Nature Neuroscience, J. Neuroscience, Neuroscience, Molecular Neurobiology and Neuropsychopharmacology*** (See the following list for details). Three additional papers will be published within the next year.
2. Nine abstracts have been presented, including one that was selected for publication in the annual meeting press book of the Society for Neuroscience Annual Meeting in November, 2003.
3. Six invited seminars have been based on the data obtained over the course of this project, including one presented at the International Conference on Disorders of Impulsivity and Amygdala Neurocircuitry in September, 2004.

REPORTABLE OUTCOMES

Peer Reviewed Papers:

1. Braga, M., Aroniadou-Anderjaska, V. and **Li, H.** :The physiological role of kainate receptors in the amygdala. An invited review paper for ***Molecular Neurobiology*** (20(2):127-141, 2004).

2. Braga, M. F., Aroniadou-Anderjaska, V., Manion, S.T., Hough, C.J., and **Li, H.:** Stress impairs α_{1A} adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala. ***Neuropsychopharmacology***. 29,45- 58, 2004

3. Chen, A., Hough, C.J. and **Li, H.**: 5-HT_2 Receptor activation facilitates synaptic plasticity via NMDA-mediated mechanism in the basolateral amygdala. ***Neuroscience.*** 119: 53-63, 2003

4. Braga, M. F., Aroniadou-Anderjaska, V., Xie, J, and **Li, H.** : Bidirectional modulation of GABA release by presynaptic glutamate 5 kainate receptors in the basolateral amygdala. ***Journal of Neuroscience***. 23(1): 442-452, 2003.

5. Rogawski, M.A., Gryder, D., Castanada, D. Yonekawa, W. Banks, M.K., **Li, H.**:Kainate receptor mediated long-term plasticity, seizures and epileptogenesis in the amygdala. ***Annal of the New York Academy of Sciences,*** 985:150-162, 2003

6. Post, R.M, Leverich, G.S., Weiss, S.R.B., Zhang, L.X., Xing, Xing, G.Q., **Li, H.** and Smith, M.: Psychosocial Stressors as predisposing factors to affective illness and PTSD: Potential neurobiological mechanisms and theoretical implications. ***Neurodevelopmental Mechanisms in Psychopathology***, Cambridge University Press, chapter 20, 491-525, 2003

7. Braga, M.F.M., Aroniadou-Anderjaska, V., Post, R.M. and **Li, H.**: Lamotrigine reduces spontaneous and evoked $GABA_A$ receptor-mediated synaptic transmission in the basolateral amygdala: Implications for its effects in seizure- and affective disorders ***Neuropharmacology,*** 42:522-529, 2002.

8. **Li, H.**, Chen, A., Xing, G., Wei, M. and Rogawski, M.A.: Kainate Receptor Mediated Heterosynaptic Facilitation in the Amygdala. ***Nature Neuroscience***, 4(6): 612-620, 2001

9. Aroniadou-Anderjaska, V., Post, R. M., Rogawski, M. A. and **Li, H.**: Input-specific LTP and depotentiation in basolateral amygdala. ***NeuroReport*** 12: 635-640, 2001

Manuscripts in preparation

1. Chen, A. and **Li, H.**: Stress impairs the 5-HT_2 receptor mediated facilitation of neuroplasticity in the basolateral amygdala.

2. Jiang X.L., and **Li, H.:** Functional alterations of alpha 2 adrenergic and serotonergic 5-HT_2 receptors in the rat basolateral amygdala after traumatic stress.

3. Braga, Maria F. M., Jiang, Xiaolong, Aroniadou-Anderjaska Vassiliki and **He Li**: Norepinephrine suppresses neuronal excitability in the basolateral amygdala.

Lay Publications

- One of our abstracts titled **"Stress impairs adrenoceptor-mediated noradrenergic facilitaiton of GABAergic transmission in the basolateral amygdala"** was submitted to the Neuroscience 2003 Annual Meeting and was one of about 600 selected (from a pool of over 16,000 submissions) by the **Public Information Committee** for inclusion in the **Annual Meeting Press Book** as a lay-language summary. Hundreds of members of the national and international media receive the Press Book before attending the annual meeting. They use the lay-language summaries contained in the book to formulate story ideas and to set up interviews with scientists whose work they find intriguing. The research paper of this work is now published in ***Neuropsychopharmacology***. 29,45-58,2004.

- Li, H.: Understanding Cellular Mechanisms of Post-traumatic Stress Disorder: Studies of Synaptic Function in Amygdala. ***Washington Psychiatric Society News*** pg.7, September-October, 2000.

Abstracts/Presentations

- M.F.Braga; X.Jiang; V.Aroniadou-Anderjaska; H.Li : Norepinephrine suppresses neuronal excitability in the basolateral amygdala. Presented at the Society for Neuroscience Meeting, (2004).

- **H.Li**[1*]; M.F.M.Braga[1]; V.Aroniadou-Anderjaska[1]; M.A.Rogawski[2] :Topiramate modulates neuronal excitability in the basolateral amygdala by selectively inhibiting GluR5 kainate receptors and acting as a positive modulator of $GABA_A$ receptors Presented in Society for Neuroscience Meeting, (2004).

- Maria F. M. Braga, Vassiliki Aroniadou-Anderjaska, Christopher J. Hough, Sean Manion and **He Li**, Stress impairs adrenoceptor-mediated noradrenergic facilitaiton of GABAergic transmission in the basolateral amygdala. Presented at the Society for Neuroscience Meeting, (2003).

- Maria F. M. Braga, and **He Li**, Topiramate enhance GABAergic transmission and blocks GluR5 kainate receptor in basolateral amygdala interneurons. Submitted to

Society for Neuroscience Meeting, (2003).

- Sean Manion, Maria F. Braga and **He Li**, Effect of traumatic stress on noradrenergic-mediated modulation of neuronal excitability and neuroplasticity in the Amygdala. Presented at the Society for Neuroscience Meeting, (2003).

- Maria F. Braga, Vassiliki Aroniadou-Anderjaska, Christopher J. Hough, Sean Manion and **He Li**: Stress impairs α1A adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala. Uniformed Services University Research Day, p46, **The President's Selected Poster Session** (2003).

- Maria F. Braga, Vassiliki Aroniadou -Anderjaska, Christopher J. Hough, Sean Manion and **He Li**: Stress impairs α1A adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala. Poster presentation in the Conference on Roots of Mental Illness in Children, Annals of the New York Academy of Sciences (2003).

- Aiqin Chen and **He Li**. Roles of Protein Kinase C and Protein Tyrosine Kinase in Theta Burst Stimulation-Induced Synaptic Potentiation in the Basolateral Amygdala. Uniformed Services University Research Day, p41, Neuroscience Session (2003).

- Sean Manion, Maria F. Braga and **He Li**, Effect of traumatic stress on noradrenergic-mediated modulation of neuronal excitability and neuroplasticity in the Amygdala. Uniformed Services University Research Day, (2003).

- Aiqin Chen, Michael A. Rogawski, Robert M. Post and **He Li** : Biphasic effects of GluR5 kainate receptor agonist on synaptic transmission in basolateral amygdala *in vitro.* (The Amygdala in Brain Function: Basic and Clinical Approaches A New York Academy of Sciences Conference, p35, 2002).
- Michael A Rogauski, Divina Gryder, Dora Castanada, Wayne Yonekawa and **He Li** : Kainate receptor mediated long-term plasticity, seizures and epileptogenesis in the amygdala. (The Amygdala in Brain Function: Basic and Clinical Approaches A New York Academy of Sciences Conference, p11, 2002).

Invited Presentations

- **He Li**: GluR5 kainate receptor and neuroplasticity in the amygdala. ***National Institute of aging***, November, 2001.

- **He Li**: GluR5 kainate receptor mediated synaptic functions in the amygdala. ***National Institute of Mental Health***, April, 2002.

- Maria F. M. Braga: Chronic Stress Causes Impairment of α_1-Adrenoceptor-mediated Modulation of GABAergic Synaptic Transmission in the Basolateral Amygdala. ***Graduate Student Colloquium and Research Day***, USUHS, 2002.

- Maria F. M. Braga: Bidirectional Modulation of GABA Release by Presynaptic GluR5 Kainate Receptors in the Basolateral Amygdala. ***USUHS Post-Doctoral Student Association Seminar Series***, 2003.

- Dr. Li was invited to give a presentation on "Plasticity in amygdala and post-traumatic stress disorder" in the A SYMPOSIUM COMMEMORATING JOHN SARVEY'S CONTRIBUTIONS TO NEUROSCIENCE AND PHARMACOLOGY (USUHS, April 2-3, 2004).

- **He Li** served as Co-Chairman of the international conference entitled **"Disorders of Impulsivity and Amygdala Neurocircuitry"**, held at the Uniformed Services University, Bethesda, MD, September 20-22, 2004. Over two hundred clinicians and researchers gathered to hear twenty-six speakers of national and international stature discuss the role of the amygdala in impulsivity disorders. Dr. Li also presented a lecture entitled "Stress impairs α_{1A} adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala." at the conference.

Employment and Research opportunities supported by this award

This award offered research opportunities for four post-doctoral fellows. One post-doctoral fellow was promoted to research assistant professor after two years training and was subsequently appointed a tenure track assistant professor at Uniformed Services University of the Health Science. The funding also supported partial salaries for one collaborator, one research associate and two Ph.D. students.

CONCLUSIONS:

The ultimate goal of this research is to acquire and provide the knowledge that is necessary for the development of novel and effective pharmacological means that will prevent or treat stress-related affective disorders, such as post-traumatic stress disorder (PTSD). The amygdala has been the focus of this study because previous studies have suggested a central role for the amygdala in stress-related emotional illnesses, both in modulating the consolidation of emotional memory and in acting as a storage site for emotional memories. Teamwork on the part of all members of the He Li laboratory has allowed us to accomplish and even surpass the goals of this project as recorded in the Statement of Work. A brief summary follows:

Our results indicate that we have successfully established and tested the inescapable tail-shock model of stress in rats and verified that long-lasting behavioral and physiological effects, known to be mediated by the amygdala, result from applying the inescapable tail-shock stress paradigm to the animals studied. We have characterized the role of glutamate 5 kainate receptors in synaptic plasticity, long term potentiation (LTP)

and low frequency induced depotentiation in the basolateral amygdala. We have also established the modulatory role of the GluR5-containing kainate receptors in inhibitory neurotransmitter release in the basolateral amygdala of control and traumatically stressed rats. By using intracellular and field recording, as well as the patch clamp technique, we have characterized the role of α_1, α_2 and β adrenoceptors and 5-HT_2 receptors in synaptic transmission, calcium signaling and neuroplasticity of the basolateral amygdala of control and stressed rats. In addition, we have characterized the facilitating effects of the serotonin type II receptor on the induction of long-term potentiation in the basolateral amygdala of control rats and rats subjected to our inescapable tail-shock paradigm. We have extended our studies to include an acute one day stress paradigm and demonstrated that both acute and repeated stressful stimuli have no significant impairment on the induction of short-term synaptic plasticity but have prolonged effects on the induction of long-term synaptic plasticity in the basolateral amygdala. The time course of resilience from such stressful stimuli is correlated with the intensity and duration of the stressors.

The information obtained from this research could be of critical importance in the development of more efficacious pharmacological interventions aimed at regulating neuronal excitability and neuroplasticity in the amygdala and thus preventing or treating stress-induced affective disorders such as PTSD. Given the chronic nature of PTSD, such interventions could bring about significant decreases in the cost to the military of treating PTSD patients.

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APPENDICES: See attached.

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The Physiological Role of Kainate Receptors in the Amygdala

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Abstract

The kainate subtype of glutamate receptors has received considerable attention in recent years, and a wealth of knowledge has been obtained regarding the function of these receptors. Kainate receptors have been shown to mediate synaptic transmission in some brain regions, modulate presynaptic release of glutamate and γ-aminobutyric acid (GABA), and mediate synaptic plasticity or the development of seizure activity. This article focuses on the function of kainate receptors in the amygdala, a brain region that plays a central role in emotional behavior and certain psychiatric illnesses. Evidence is reviewed indicating that postsynaptic kainate receptors containing the glutamate receptor 5 kainate receptor (GLU_{k5}) subunit are present on interneurons and pyramidal cells in the basolateral amygdala and mediate a component of the synaptic responses of these neurons to glutamatergic input. In addition, GLU_{k5}-containing kainate receptors are present on presynaptic terminals of GABAergic neurons, where they modulate the release of GABA in an agonist concentration-dependent, bidirectional manner. GLU_{k5}-containing kainate receptors also mediate a longlasting synaptic facilitation induced by low-frequency stimulation in the external capsule to the basolateral nucleus pathway, and they appear to be partly responsible for the susceptibility of the amygdala to epileptogenesis. Taken together, these findings have suggested a prominent role of GLU_{k5}-containing kainate receptors in the regulation of neuronal excitability in the amygdala.

Index Entries: Kainate receptors; GLU_{k5}; amygdala; excitatory synaptic transmission; inhibitory synaptic transmission; synaptic plasticity; long-term potentiation; epilepsy; emotional memory; mood disorders.

Introduction

Fast excitatory neurotransmission in the vertebrate central nervous system (CNS) is mediated primarily by glutamate. Based on pharmacological studies using selective agonists, ionotropic glutamate receptors have been divided into three major classes of receptor subtypes: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors. Subsequent molecular cloning of subunits

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that form glutamate receptors has confirmed the validity of this pharmacological subdivision and has greatly enhanced our understanding of their functional properties.

Kainate receptors consist of five different subunits, namely, GLU_{k5}, GLU_{k6}, GLU_{k7}, GLU_{k1}, and GLU_{k2} (reviewed in refs. *1* and *2*). GLU_{k5-7} subunits form homo- and heteromeric functional channels when expressed in heterologous systems *(3–8)*. GLU_{k1} and GLU_{k2} subunits do not form functional homomers in the same systems but generate functional receptors with distinct physiological properties when combined with GLU_{k5}, GLU_{k6}, or GLU_{k7} subunits *(6,9,10)*. Consequently, a large number of distinct kainate receptor subtypes could be assembled based on the combinatorial possibilities of these five distinct subunits. In addition, kainate receptor subunits are subjected both to alternative splicing and RNA editing, which significantly increase the number of subunit isoforms. Alternative splicing has been reported for GLU_{k5}, GLU_{k6}, and GLU_{k7} subunits *(3,4,6,11,12)*, but the role of the different splice variants is unknown. Posttranscriptional messenger RNA (mRNA) editing has been described for the GLU_{k5} and GLU_{k6} subunits at the Q/R site of the M2 domain *(13,14)*, which decreases the permeability to calcium *(5,15)* and transforms the rectification properties of these receptors from inwardly rectifying to linear or slightly outwardly rectifying *(14,16–20)*. GLU_{k6} also can undergo further editing at two additional sites in the M1 domain *(21)*; however, the role of the M1 editing sites remains unknown.

Although little is known about the precise subunit composition of native kainate receptors, their potential compositional diversity is evident by the numerous, distinct physiological roles that these receptors seem to play in the CNS. Kainate receptors have been shown to mediate fast excitatory synaptic transmission *(22–26)*, modulate transmitter release at both excitatory and inhibitory synapses (reviewed in refs. *27–29*), and are involved in short- and long-term synaptic plasticity mechanisms (reviewed in refs. *1* and *2*).

Although kainate receptor subunit genes are widely expressed throughout the brain *(30)*, it appears that in different brain regions, kainate receptors may have different functions. Their function may ultimately depend on their cellular and subcellular localization, subunit composition and stoichiometry, and density. This article addresses the functional roles of kainate receptors in the amygdala, a brain region that plays a central role in all aspects of emotional behavior, such as emotional learning and memory functions *(31–34)*, responses to psychological stress *(35–37)*, as well as pathophysiological conditions such as those associated with affective disorders *(38–42)* or temporal lobe epilepsy *(43)*.

Kainate Receptors are Highly Expressed in the Amygdala

The amygdaloid complex is a group of more than 10 nuclei that are located in the midtemporal lobe and have extensive internuclear and intranuclear connections *(44)*. The amygdala receives information from all sensory modalities via glutamatergic excitatory inputs from the cerebral cortex, the thalamus, and other subcortical brain regions *(44,45)*. Glutamate is also the major excitatory neurotransmitter in intra-amygdala circuits *(46–51)*. It has been shown that glutamatergic synapses in the amygdala express NMDA, AMPA, and kainate receptors (for a review, *see* ref. *45*).

In situ hybridization studies have revealed that certain kainate receptor subunits are highly expressed in the amygdala (Fig. 1). Thus, mRNA levels of GLU_{k5}, GLU_{k6}, and GLU_{k2} subunits are high in most regions of the amygdala *(52)*. In particular, the GLU_{k5} subunit is higher in the amygdala than in the hippocampus, and it is mainly concentrated in the basolateral and medial nuclei *(52,53)*.

The heavy expression of kainate receptors in the amygdala may imply a prominent physiological role of these receptors in this brain region. Indeed, there is evidence that kainate

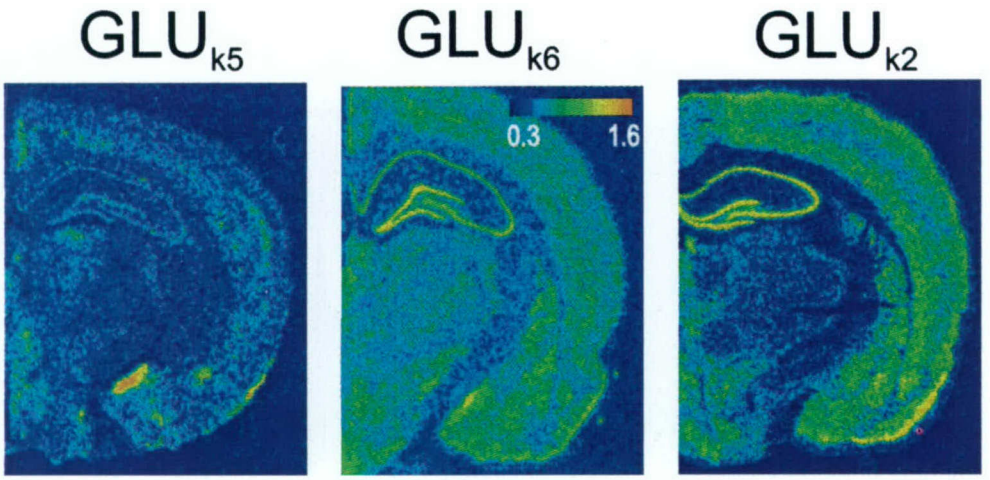

Fig. 1. Pseudocolor images of GLU_{k5}, GLU_{k6}, and GLU_{k2} mRNA expression, as revealed by *in situ* hybridization in rat brain coronal sections at the level of the amygdala. Although GLU_{k6} and GLU_{k2} mRNA signal is strongest in the hippocampus, GLU_{k5} mRNA expression is highest in the amygdala.

receptors in the amygdala (a) mediate a component of excitatory synaptic transmission; (b) modulate the release of GABA in interneuron to pyramidal cell synapses; (c) play an important role in certain forms of amygdalar synaptic plasticity; and (d) are significantly involved in certain pathophysiological conditions of the amygdala, such as temporal lobe epilepsy.

Postsynaptic GLU_{k5} Kainate Receptors Mediate Excitatory Synaptic Transmission in the Amygdala

Early evidence for kainate receptor-mediated excitatory synaptic responses came from observations in the hippocampal mossy fiber synapses *(22,54)*. These studies became possible when pharmacological tools capable of selectively blocking AMPA or kainate receptors became available *(22,54–57)*. Subsequently, kainate receptor-mediated synaptic responses have been reported in cerebellar Golgi cells *(58)*, at thalamocortical synapses *(26)*, in sensory fiber-dorsal horn neurons in the spinal cord *(59)*, and in the basolateral nucleus of the amygdala (BLA; refs. *25* and *53*).

Most studies on the function of kainate receptors in the amygdala have focused on the BLA. The BLA, along with the lateral nucleus, is the entry site for afferent inputs to the amygdala *(45)*. In the BLA, the bulk (about 70%; ref. *25*) of the glutamatergic excitatory postsynaptic responses is mediated by AMPA receptors. However, using selective pharmacological antagonists, Li and Rogawski (1998) first demonstrated that a component (about 30%) of the excitatory postsynaptic potential (EPSP) evoked by stimulation of the external capsule in BLA neurons is mediated by kainate receptors *(25)*. Therefore, it was concluded that a component of the EPSP was resistant to NMDA receptor antagonists and to the AMPA receptor-selective, allosteric antagonists GYKI 52466 and GYKI 53655. This component was blocked by the GLU_{k5}-selective kainate receptor antagonist LY293558, suggesting that it was mediated by GLU_{k5}-containing kainate receptors (Fig. 2). As in the hippocampus, the GLU_{k5}-mediated EPSP showed a remarkable dependence on the stimulation frequency. Increasing the stimulation

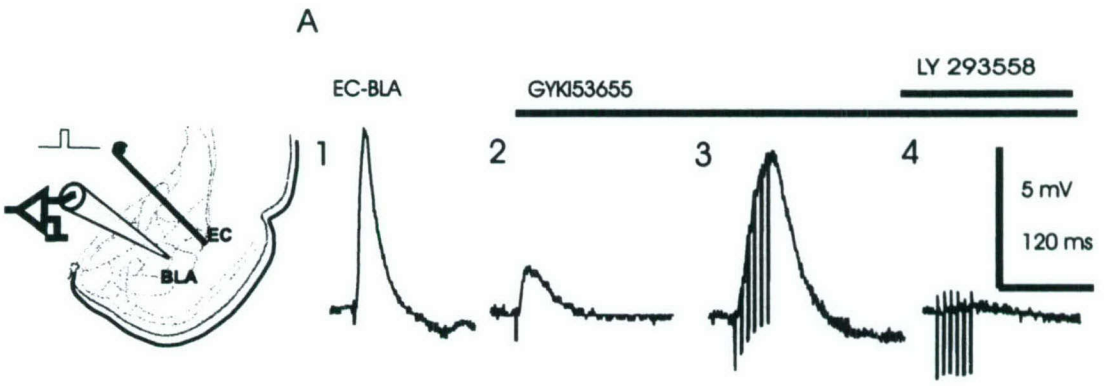

Fig. 2. GLU_{k5} receptors mediate a component of the EPSP evoked in BLA neurons by stimulation of the external capsule. The AMPA receptor antagonist GYKI-53655 (50 μ*M*) reduced the EPSP evoked by single-pulse stimulation of the external capsule *(2)*. High-frequency stimulation (six pulses at 100 Hz) substantially increased the magnitude of the residual, GYKI 53655-resistant EPSP, which was subsequently blocked by the GLU_{k5} antagonist LY293558 (10 μ*M*). The slice medium contains the NMDA receptor antagonist APV (100 μ*M*) and the $GABA_A$ receptor antagonist bicuculline (10 μ*M*). The recording electrode contains 50 m*M* of QX-314.

frequency of the external capsule produced a large increase in the amplitude of the kainate receptor-mediated synaptic responses (Fig. 2).

Subsequently, Braga et al. *(53)* found that a specific GLU_{k5} kainate receptor agonist enhances the frequency and amplitude of Tetrodotoxin (TTX)-sensitive, spontaneous GABAergic currents (inhibitory postsynaptic currents; IPSCs) recorded from BLA pyramidal cells (Fig. 3B). This observation suggested that GLU_{k5} kainate receptor activation depolarizes inhibitory interneurons. To determine whether postsynaptic GLU_{k5} kainate receptors are actually present on BLA interneurons, excitatory postsynaptic currents (EPSCs) evoked by electric stimulation of the external capsule (three shocks delivered at 100 Hz every 10 s) were recorded from identified BLA interneurons in the presence of GYKI 53655 (50 μ*M*), phosphonovaleric acid (D-APV) (50 μ*M*), bicuculline (10 μ*M*), and SCH50911 (20 μ*M*) to block AMPA, NMDA, $GABA_A$, and $GABA_B$ receptors, respectively. These evoked EPSCs were completely blocked by bath application of LY293558, suggesting that they were mediated by GLU_{k5} kainate receptors (Fig. 3A). GLU_{k5} kainate receptor-mediated EPSCs were also recorded from BLA pyramidal neurons *(60)*. Thus, it appears that in the BLA, GLU_{k5} kainate receptors are present on somatodendritic regions of both pyramidal cells and interneurons and mediate a component of the evoked EPSCs.

Bidirectional Modulation of GABA Release by Presynaptic GLU_{k5} Kainate Receptors in the BLA

In addition to mediating excitatory synaptic transmission in some brain regions, kainate receptors have been shown to modulate the release of glutamate and GABA (reviewed in refs. *27* and *28*). In both excitatory and inhibitory synapses, kainate receptors initially were found to depress neurotransmitter release *(28)*. More recent studies demonstrated that kainate receptor activation can also facilitate transmitter release *(61–64)*.

In the BLA, Braga et al. *(53)* showed that GLU_{k5} kainate receptors were present on presynaptic GABAergic terminals contacting

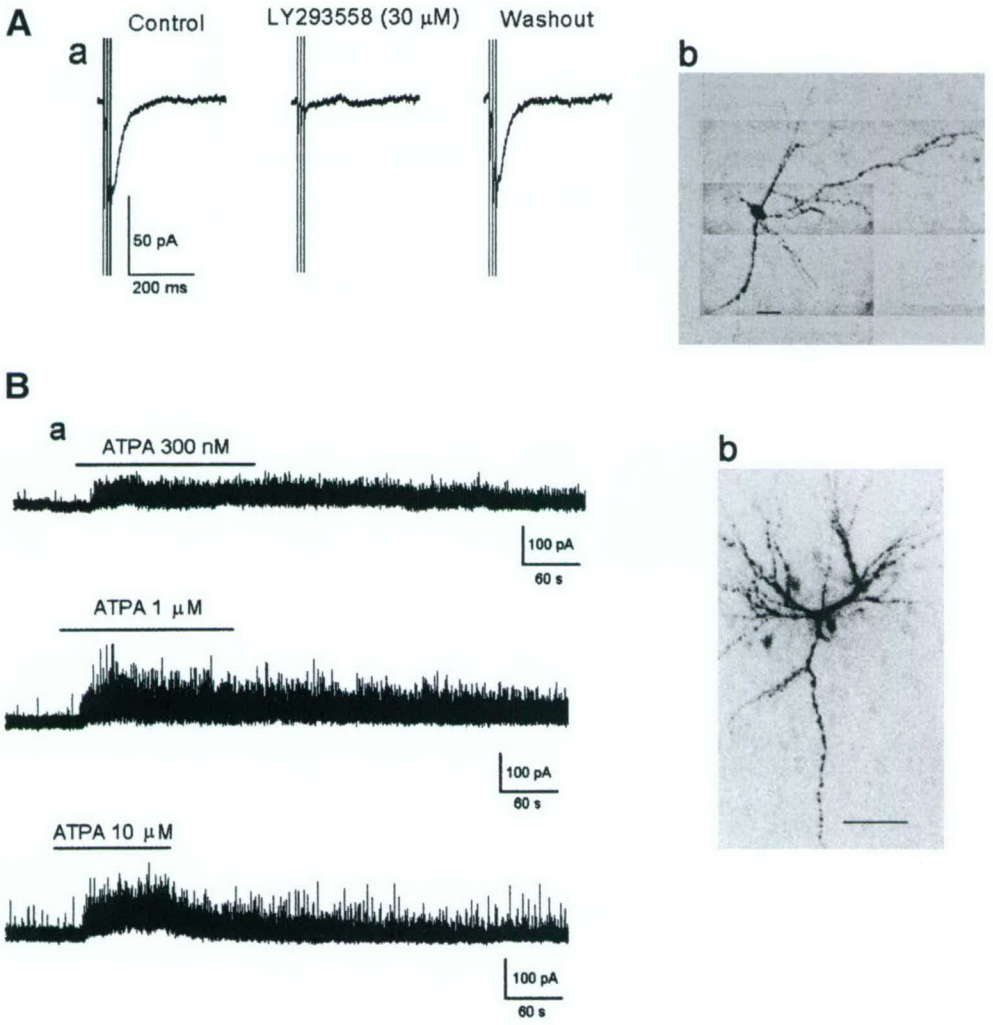

Fig. 3. Excitation of BLA interneurons via GLU_{k5} kainate receptors. **(Aa)** GLU_{k5} kainate receptors mediate a component of the synaptic responses of BLA interneurons. Excitatory postsynaptic currents (Vh–60 mV) recorded from a BLA interneuron in the presence of GYKI 53655 (50 μ*M*), D-APV (50 μ*M*), bicuculline (10 μ*M*), and SCH50911 (20 μ*M*). Electrical stimulation was applied to the external capsule (three shocks delivered at 100 Hz every 10 s). The EPSC was blocked by the GLU_{k5} antagonist LY293558. A photomicrograph of the interneuron recorded in (a) is shown (b) (scale bar: 50 μm). **(Ba)** Activation of GLU_{k5} kainate receptors increases spontaneous activity of BLA interneurons. Effects of different concentrations of ATPA on spontaneous IPSCs recorded from the soma of three different BLA pyramidal neurons (Vh +10 mV). A photomicrograph of one of these neurons is shown in **(Bb)** (scale bar: 100 μm).

pyramidal cells and that activation of these receptors bidirectionally modulated the release of GABA in an agonist concentration-dependent manner. Therefore, low concentrations of the specific GLU_{k5} kainate receptor agonist ATPA or glutamate (0.3 and 5 μ*M*, respectively) potentiated evoked GABA release, whereas high concentrations of the agonists (10 μ*M* of

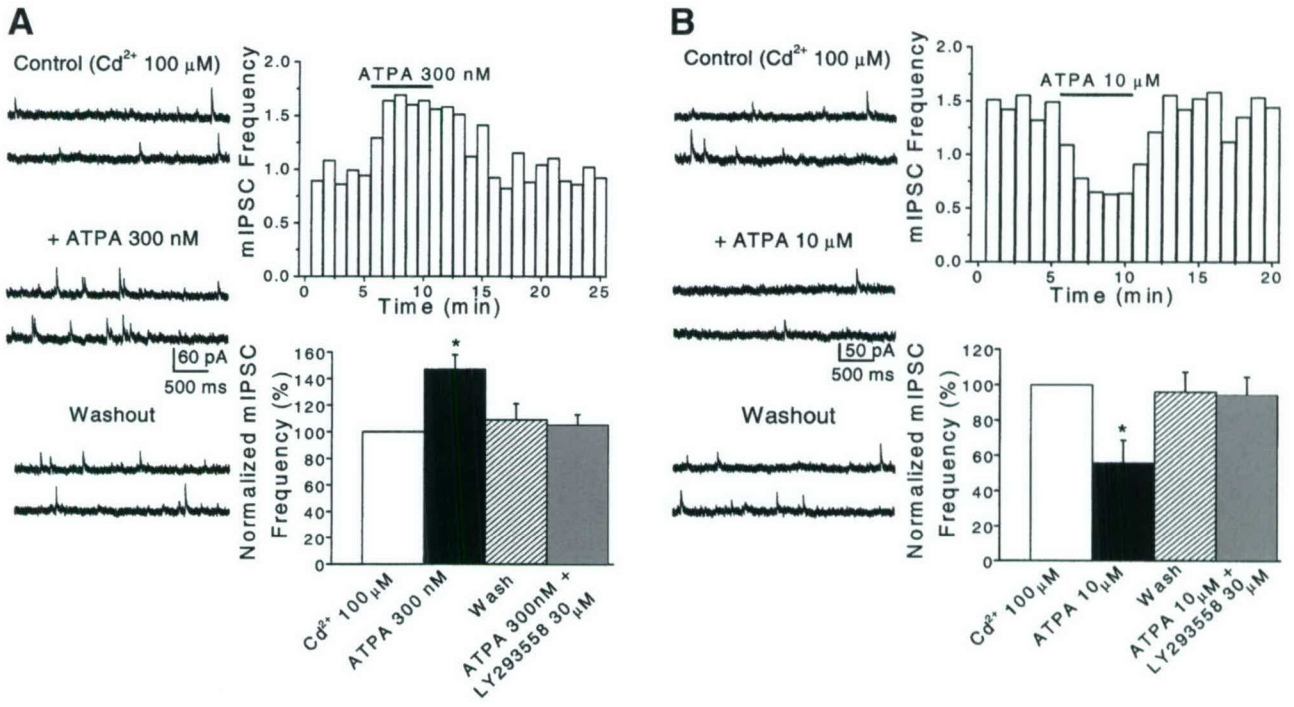

Fig. 4. Dose-dependent, bidirectional modulation of the frequency of miniature GABAergic currents by the GLU_{k5} agonist ATPA. Traces in **(A)** and **(B)** are samples of miniature IPSCs (mIPSCs) recorded from two different BLA pyramidal neurons before, during, and after application of 300 n*M* (panel A) or 10 µ*M* (panel B) of ATPA in the presence of Cd^{2+} (100 µ*M*), TTX (1 µ*M*), GYKI 53655 (50 µ*M*), D-APV (50 µ*M*), and SCH50911 (20 µ*M*) at a holding potential of +10 mV. Top plots show the effects of 300 n*M* (panel A) and 10 µ*M* (panel B) of ATPA on the mean frequency of mIPSCs as a function of time (bin = 60 s). Bottom bar graphs show pooled data (mean ± standard error of the mean [SEM]). At 300 n*M* (panel A), ATPA increased the frequency of mIPSCs ($n = 6$, $^*p < 0.05$). At 10 µ*M* (panel B), ATPA produced a marked reduction in the frequency of mIPSCs ($n = 3$, $^*p < 0.05$). For each cell, mIPSC frequency was normalized to the value of mean mIPSC frequency before application of ATPA. Coapplication of LY293558 (30 µ*M*) prevented the effects of ATPA.

ATPA or 200 µ*M* of glutamate) depressed it. These effects were unrelated to activation of $GABA_B$ or group I metabotropic glutamate receptors, because they persisted in the presence of SCH 50911 and CPCCOEt. Low concentrations of ATPA or glutamate also increased the frequency of miniature IPSCs, whereas high concentrations of these agonists reduced it (Fig. 4). The effects of the GLU_{k5} kainate receptor agonists on the TTX-insensitive release of GABA did not require activation of voltage-dependent Ca^{2+} channels, $GABA_B$ receptors, or group I metabotropic glutamate receptors. The same study provided evidence that endogenous glutamate gains access to presynaptic GLU_{k5} kainate receptors that are present on inhibitory terminals and tonically facilitates evoked GABA release.

These findings led to the hypothesis that the terminals of GABAergic neurons in the BLA contain two subtypes of GLU_{k5}-bearing kainate receptors, which have different affinities to their agonists and activate different mechanisms of action. Based on their affinity for [^{3}H]kainate, kainate receptor subunits can be divided into low-affinity (GLU_{k5}, GLU_{k6}, and GLU_{k7}) and high-affinity (GLU_{k1} and GLU_{k2}) subunits *(65)*. The BLA expresses high levels of

the GLU_{k6} and GLU_{k2} subunit mRNAs in addition to GLU_{k5} *(52)*. There is evidence that the GLU_{k5} subunit can form functional kainate receptors with GLU_{k6} or GLU_{k2} subunits, and both GLU_{k5}/GLU_{k6} and GLU_{k5}/GLU_{k2} kainate receptors are sensitive to ATPA *(8)*. Therefore, a GLU_{k5}/GLU_{k2} and a GLU_{k5}/GLU_{k6} subunit combination could mediate the facilitation and inhibition of GABAergic transmission in the BLA, respectively. Consistent with the view that a GLU_{k5}/GLU_{k6} subunit combination may mediate the suppression of GABAergic transmission in the BLA, Mulle et al. *(66)* found that kainate-induced suppression of evoked IPSCs in the hippocampus is mediated by heteromeric kainate receptors composed of both GLU_{k5} and GLU_{k6} subunits.

The intracellular mechanisms to which these presynaptic GLU_{k5} receptors are coupled remain to be elucidated. In the hippocampus, evidence exists for the participation of both metabotropic and ionotropic cascades following the activation of kainate receptors *(62,66–69)*. The agonist concentration-dependent, bidirectional modulation of GABA release via presynaptic GLU_{k5} kainate receptors in the BLA suggests a significant role of glutamate diffusion in the regulation of neuronal excitability in this brain region. Low concentrations of extracellular glutamate escaping from excitatory synapses during tonic or low-level activity of excitatory pathways in the BLA can be expected to facilitate GABAergic transmission. Considering the central role of the amygdala and the BLA in particular, in fear-conditioning and consolidation of emotional memories *(70)*, such facilitation of GABAergic transmission may prevent or dampen excitation of the amygdala during external or internal stimuli that have only modest emotional significance. In contrast, in response to intense emotional stimuli that produce strong excitation of the amygdala, the amount of glutamate released may reach sufficiently high extrasynaptic concentrations to activate the low-affinity GLU_{k5} kainate receptors on GABAergic terminals, inhibiting evoked GABAergic transmission. This effect could further enhance overactivity in the amygdala during intense emotional stimuli and perhaps facilitate the "registration" of the memory trace representing the emotional event. In that respect, this GLU_{k5}-mediated disinhibitory effect of glutamate may play an important role in synaptic plasticity and memory formation in the amygdala, as well as in the development of certain stress-related affective disorders such as posttraumatic stress syndrome.

Kainate Receptors Mediate a Form of Synaptic Plasticity in the Amygdala

Synaptic plasticity phenomena such as long-term potentiation (LTP) and long-term depression (LTD) are believed to be cellular mechanisms that underlie learning and memory processes *(71–76)*. In all brain regions examined to date, the intracellular events that induce LTP or LTD are triggered by a rise in intracellular calcium postsynaptically and, in some synapses, presynaptically *(72,77–81)*. In most forms of LTP and LTD, the mechanism by which intracellular free calcium increases is the influx of calcium via postsynaptic NMDA receptors *(77,81–87)*. However, synaptic plasticity that does not require NMDA receptor activation has also been reported in many brain regions *(79,87–92)*.

Various forms of synaptic plasticity also have been described in the amygdala *(93–100)*. A novel form of synaptic plasticity, in which low-frequency stimulation (1 Hz for 15 min) of the external capsule induces a long-lasting synaptic facilitation of EPSPs recorded from BLA neurons *(51)*, was shown to be mediated by GLU_{k5} kainate receptors *(52)*. Thus, induction of this form of synaptic potentiation (low-frequency-induced facilitation [LFIF]) was blocked by antagonists that were selective for GLU_{k5} kainate receptors (LY377770 and LY382884) but not by antagonists of NMDA (100 μM of APV), AMPA (50 μM of GYKI53655), or group I metabotropic (20 μM of CPCCOEt) glutamate receptors. Furthermore, a similar form of lasting potentiation was induced by brief (10 min)

exposure of the amygdala to the GLU_{k5}-selective agonist ATPA (20 μ*M*). An increase in intracellular calcium was necessary for the induction of the GLU_{k5} kainate receptor-mediated LFIF. Potentiation was expressed in both the NMDA and the AMPA/kainate receptor-mediated components of the EPSPs. Interestingly, potentiation was not restricted to the fibers stimulated during the induction period (homosynaptic potentiation) but rather was generalized to other converging pathways (heterosynaptic potentiation; ref. *52*).

The mechanisms by which GLU_{k5} kainate receptors mediate the induction of LFIF and the mechanisms of heterosynaptic spread of this form of synaptic facilitation remain to be elucidated. GLU_{k5} kainate receptors can be permeable to calcium, particularly when they contain unedited kainate receptor subunits *(65)*. About 30% of the GLU_{k5} subunits present in the BLA are in the unedited form *(52)*, and, therefore, they may participate in forming calcium permeable kainate receptors that contribute to synaptic plasticity. It is not known whether the GLU_{k5}-containing receptors that mediate LFIF are present postsynaptically or presynaptically. As mentioned earlier, there is electrophysiological evidence that GLU_{k5} receptors are present on somatodendritic regions of both BLA pyramidal cells and interneurons. It remains to be determined whether they are also present on glutamatergic presynaptic terminals of afferent pathways. The enhancement of both the NMDA and AMPA/kainate components of the EPSP may suggest involvement of presynaptic mechanisms.

Kainate receptors desensitize rapidly *(101)*. This may be one reason that they generally do not contribute significantly to the induction of LTP by high-frequency stimulation. However, during low-frequency stimulation, these receptors may have sufficient time to recover from the desensitized state and thus contribute to postsynaptic depolarization and calcium influx.

In the hippocampus, low-frequency stimulation induces LTD *(102)*. One reason for this difference between the hippocampus and the external capsule to BLA pathway may be that the BLA has a substantially higher concentration of GLU_{k5} kainate receptors, and, therefore, more calcium may enter postsynaptically (and/or presynaptically) during low-frequency stimulation, resulting in synaptic potentiation rather than depression.

Kainate Receptors and Temporal Lobe Epilepsy

The amygdala plays a central role in temporal lobe epilepsy *(43,103)*. It is a key structure in the generation of seizures as well as in the spread of limbic seizure activity through its connections with the entorhinal cortex and hippocampus *(103)*. Little is known about the mechanisms that underlie the amygdala's susceptibility to epileptogenesis. However, kainate receptors appear to play a significant role, because a single injection of kainic acid (a preferential kainate receptor agonist) into the amygdala produces cell damage and elicits chronic, spontaneous, recurrent epileptiform activity similar to that observed in human temporal lobe epilepsy. Recent evidence suggests that GLU_{k5} kainate receptors, in particular, may play an important role in the vulnerability of the amygdala *(43)*. Thus, ATPA induces spontaneous epileptiform bursting in amygdala slices and limbic status epilepticus when infused into the rat amygdala. The effects of ATPA are blocked by the GLU_{k5} kainate receptor antagonist LY293558. Additional evidence that GLU_{k5} kainate receptors are involved in the generation of epileptic activity in the amygdala came from the findings that the anticonvulsant topiramate inhibits GLU_{k5} kainate receptor-mediated synaptic currents in the BLA *(60,104)*. Topiramate-induced inhibition of GLU_{k5} kainate receptors on somatodendritic regions of BLA pyramidal cells *(60)* would suppress seizure activity by suppression of excitatory transmission. Topiramate also inhibited GLU_{k5} kainate receptor activity on BLA GABAergic neurons *(104)*. Inhibition of GLU_{k5}

kainate receptors on somatodendritic regions of GABAergic cells by topiramate could reduce GABAergic activity. However, GLU_{k5} kainate receptors also are present on GABAergic terminals, where they suppress GABA release when extracellular concentrations of glutamate are increased, as during epileptic activity. Topiramate would relieve the GLU_{k5}-mediated suppression of GABA release, thus facilitating inhibitory transmission. Therefore, these results *(60,104)* suggest that topiramate may protect against seizures, at least in part, through suppression of GLU_{k5} kainate receptor activity. In this regard, it is interesting that topiramate has produced promising results in the treatment of certain psychiatric illnesses *(105–107)*. The issue of whether a suppression of GLU_{k5} activity is involved in the effectiveness of topiramate in the treatment of these disorders is an attractive possibility that deserves to be explored.

What could be the mechanisms by which GLU_{k5} kainate receptor agonists induce epileptic activity in the amygdala? As discussed earlier, current evidence suggests that GLU_{k5}-containing kainate receptors are present on somatodendritic sites of both pyramidal cells *(60)* and interneurons *(53,104)*, as well as on presynaptic terminals of GABAergic interneurons *(53)*. The action of GLU_{k5} agonists on somatodendritic regions of interneneurons depolarizes these cells, enhancing spontaneous GABA release, which would suppress amygdalar excitability. In contrast, the action of the GLU_{k5} agonists on somatodendritic regions of pyramidal cells depolarizes these cells, increasing glutamate release, which would enhance amygdalar excitability. At the same time, the GLU_{k5} kainate receptor agonists are acting at GABAergic presynaptic terminals. When agonist concentrations are low, evoked GABA release is enhanced, which favors suppression of pyramidal cell excitability. In contrast, when agonist concentrations are sufficiently high, evoked GABA release is suppressed, which favors an enhancement of neuronal excitability. Field potential recordings have indicated that the net effect of low-level activation of GLU_{k5} kainate receptors (1 μM of ATPA in the slice medium) is a suppression in the overall neuronal excitability in the BLA, whereas the net effect of strong activation of GLU_{k5} kainate receptors (10 μM of ATPA) is an enhancement in overall neuronal excitability and generation of epileptiform activity (Aroniadou-Anderjaska et al., unpublished observations, 2003). The effects of GLU_{k5} kainate receptor activation are summarized in Fig. 5.

Perspectives

Understanding the physiology of the amygdala is central to understanding the neurobiological mechanisms underlying emotional behavior as well as psychiatric illnesses such as affective disorders (including stress-related affective disorders, whose incidence has substantially increased in recent years) or temporal lobe epilepsy. Knowledge of the mechanisms that regulate neuronal excitability in the amygdala is imperative in understanding the pathophysiology of these diseases as well as in the discovery of new, effective treatment strategies. The prominent presence of kainate receptors in the amygdala suggests that these receptors may play a significant role in the function of the amygdala. As discussed in this article, recent evidence indicates that GLU_{k5}-containing kainate receptors play an important role in the regulation of amygdalar excitability. However, numerous questions remain to be answered before a complete view emerges regarding the functions of GLU_{k5} kainate receptors in the amygdala. For example, it remains to be determined whether GLU_{k5} kainate receptors are present on excitatory synaptic terminals, regulating glutamate release. The presence of low- and high-affinity GLU_{k5}-containing kainate receptors on GABAergic terminals must be confirmed by further studies, and the opposing intracellular signaling pathways that these receptors activate, which produce suppression or enhancement of GABA release, remain to be investigated. The precise mechanisms by which GLU_{k5} kainate receptors

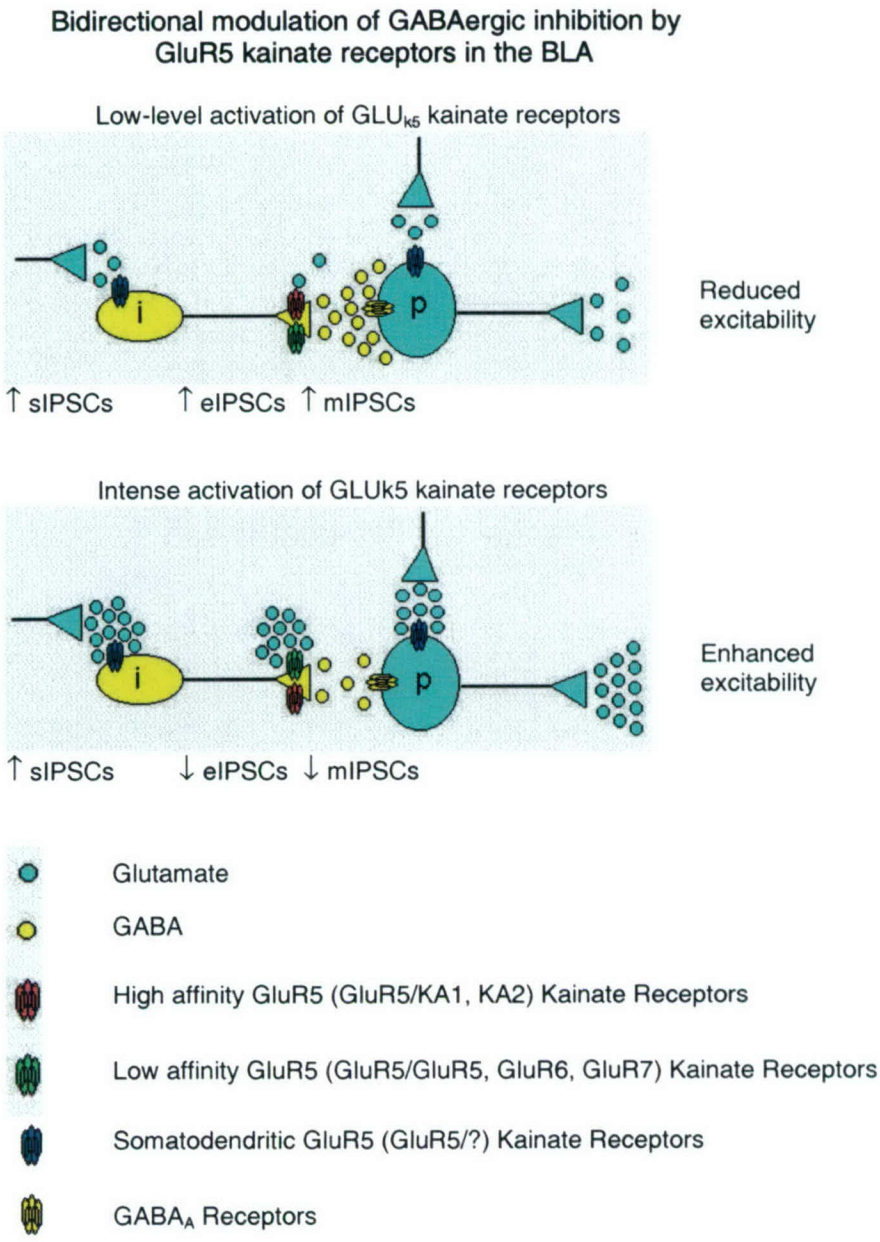

Fig. 5. Schematic representation of the agonist concentration-dependent bidirectional modulation of neuronal excitability by GLU$_{k5}$ receptors, in the BLA. Physiological studies have suggested the presence of GLU$_{k5}$-containing kainate receptors on somatodendritic sites of both pyramidal cells and interneurons as well as on presynaptic terminals of GABAergic interneurons. GABAergic terminals appear to carry two subtypes of GLU$_{k5}$-containing kainate receptors, which have different affinities for glutamate and activate opposing mechanisms of action. Low concentrations of GLU$_{k5}$ kainate receptor agonists depolarize both pyramidal cells and interneurons (via somatodendritic receptors) and increase evoked GABA release (and mIPSCs) via activation of the high-affinity, presynaptic GLU$_{k5}$ kainate receptors. The result is a substantial increase in GABA release, which may suppress excitability of the BLA neuronal network. High concentrations of GLU$_{k5}$ kainate receptor agonists again depolarize both interneurons and pyramidal cells and suppress evoked GABA release (and mIPSCs) via activation of the low-affinity, presynaptic GLU$_{k5}$ kainate receptors. The result is likely an enhancement in the excitability of the BLA neuronal network. These hypotheses regarding the net effects of low or high agonist concentrations have been supported by field potential recordings (*see* text). p, pyramidal cell; i, interneuron; eIPSC, evoked inhibitory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; sIPSP, spontaneous inhibitory postsynaptic current.

mediate synaptic plasticity in the BLA must also be delineated. The role of other subtypes of kainate receptors in the amygdala's physiology and the composition and stoichiometry of native kainate receptors await further study.

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Stress Impairs α_{1A} Adrenoceptor-Mediated Noradrenergic Facilitation of GABAergic Transmission in the Basolateral Amygdala

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Intense or chronic stress can produce pathophysiological alterations in the systems involved in the stress response. The amygdala is a key component of the brain's neuronal network that processes and assigns emotional value to life's experiences, consolidates the memory of emotionally significant events, and organizes the behavioral response to these events. Clinical evidence indicates that certain stress-related affective disorders are associated with changes in the amygdala's excitability, implicating a possible dysfunction of the GABAergic system. An important modulator of the GABAergic synaptic transmission, and one that is also central to the stress response is norepinephrine (NE). In the present study, we examined the hypothesis that stress impairs the noradrenergic modulation of GABAergic transmission in the basolateral amygdala (BLA). In control rats, NE (10 μM) facilitated spontaneous, evoked, and miniature IPSCs in the presence of β and α_2 adrenoceptor antagonists. The effects of NE were not blocked by α_{1D} and α_{1B} adrenoceptor antagonists, and were mimicked by the α_{1A} agonist, A61603 (1 μM). In restrain/tail-shock stressed rats, NE or A61603 had no significant effects on GABAergic transmission. Thus, in the BLA, NE acting via presynaptic α_{1A} adrenoceptors facilitates GABAergic inhibition, and this effect is severely impaired by stress. This is the first direct evidence of stress-induced impairment in the modulation of GABAergic synaptic transmission. The present findings provide an insight into possible mechanisms underlying the antiepileptogenic effects of NE in temporal lobe epilepsy, the hyperexcitability and hyper-responsiveness of the amygdala in certain stress-related affective disorders, and the stress-induced exacerbation of seizure activity in epileptic patients.

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INTRODUCTION

Many components of the biological response to emotional stressors are of vital importance in enabling the individual to cope with stress. However, it is well known that excessive or repeated stress can have detrimental effects on health that are often associated with functional alterations in the systems involved in the stress response (Vermetten and Bremner, 2002; Vanitallie, 2002; McEwen, 2002; Pawlak *et al*, 2003). The amygdala is a key component of the brain's neuronal network that determines the emotional significance of external events (LeDoux, 1992; Davis, 1994; Breiter *et al*, 1996; Schneider *et al*, 1997; LaBar *et al*, 1998; Buchel *et al*, 1998; Whalen *et al*, 1998; Baird *et al*, 1999; Davidson *et al*, 1999; Davidson and Slagter, 2000; Buchel and Dolan, 2000). Via efferent pathways to the hypothalamus, the amygdala can also trigger the neuroendocrine cascades that are part of the stress response (Habib *et al*, 2001; Pitkänen, 2000; Davis, 1992) and via reciprocal connections with the cerebral cortex and limbic structures, it modulates the orchestration of the behavioral response (Goldstein *et al*, 1996; Pitkanen *et al*, 2000). Therefore, understanding the changes in the amygdala's physiology and function induced by stress is critical in understanding the pathophysiology of stress, and may aid the development of new therapeutic strategies for the prevention and treatment of stress-related, affective disorders.

Different lines of evidence point to the possibility that the function of the GABAergic system may be impaired by stress. First, in a number of brain regions, benzodiazepine receptor binding is altered by stress (Lippa *et al*, 1978; Medina *et al*, 1983; Miller *et al*, 1987; Weizman *et al*, 1989; Bremner *et al*, 2000). Second, in certain stress-related psychiatric disorders, the amygdala exhibits higher than normal levels of basal activity (Abercrombie *et al*, 1998; Drevets, 1999), or exaggerated responses to fearful stimuli (Rauch *et al*, 2000; Villarreal and King, 2001). Since the GABAergic system is a primary regulator of neuronal excitability, pathophysiological changes in GABAergic transmission may underlie the amygdala's hyper-responsiveness and hyperexcitability in these emotional disorders.

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Third, many psychotropic drugs that are effective in the treatment of emotional disorders target or influence GABAergic transmission. Fourth, stress exacerbates the frequency of seizures in epileptic patients (Temkin and Davis, 1984; Frucht *et al*, 2000). However, there is no direct evidence, so far, for stress-induced impairment in GABAergic synaptic transmission.

One of the modulators of GABA release is norepinephrine (NE), which is also central to the stress response. During stress, there is a dramatic increase in noradrenergic activity following the peripheral release of epinephrine from the adrenal glands, and the central release of NE, predominantly from the locus ceruleus (Stanford, 1995; Bremner *et al*, 1996). The amygdala receives dense noradrenergic afferents from the locus ceruleus (Pitkänen, 2000), as well as from other brain regions such as the nucleus of the solitary tract (Pitkänen, 2000; Clayton and Williams, 2000; Williams *et al*, 2000). During stress, there is a strong enhancement of NE release in the amygdala (Galvez *et al*, 1996; Stanford, 1995; Quirarte *et al*, 1998; Tanaka *et al*, 2000). The short- and long-term consequences of stress-induced excessive NE release on amygdala's physiology are unknown.

NE modulates GABAergic inhibition primarily via the α_1 subtype of adrenergic receptors (Gellman and Aghajanian, 1993; Alreja and Liu, 1996; Bergles *et al*, 1996; Kawaguchi and Shindou, 1998). There is evidence suggesting that α_1 adrenoceptors are affected by stress. Thus, chronic stress, in rats, reduces the expression of these receptors in the hypothalamus and brain stem (Miyahara *et al*, 1999). α_1 adrenoceptor binding is also reduced in depressed patients (Crow *et al*, 1984; Gross-Isseroff *et al*, 1990), and blockade of these receptors in rats increases depressive behavior (Stone and Quartermain, 1999). The physiological implications of stress-induced reduction in α_1 adrenoceptor activity are not known.

In the present study, we investigated whether NE modulates GABAergic transmission in the basolateral nucleus of the amygdala (BLA), and if so, whether the noradrenergic modulation of the GABAergic transmission is altered by exposure to stress. We studied the BLA because this amygdala region is heavily involved in the processing of emotional experiences, as it receives both direct and indirect thalamic and cortical inputs and is extensively interconnected with the prefrontal/frontal cortex and the hippocampus (Pitkänen, 2000). Furthermore, it appears that the BLA selectively (among the different amygdala nuclei) modulates the consolidation of emotional memories (Cahill and McGaugh, 1998; Ferry *et al*, 1999). Our results show that NE facilitates spontaneous, evoked, and action potential-independent, quantal GABA release in the BLA via the α_{1A} subtype of adrenergic receptors, and that exposure to stress severely impairs this α_1 adrenoceptor-mediated facilitation of GABA release.

METHODS

Animals and Stress Protocol

All animal experiments were performed in accordance with our institutional guidelines after obtaining the approval of the Institutional Animal Care and Use Committee (IACUC). Male, Sprague–Dawley rat pups were received with their mother at postnatal day (PND) 17, and housed in a climate-controlled environment on a 12 h light/dark cycle (lights on at 0700). On PND 21, the rats were weaned, assigned numbers, and randomly divided into control and stressed groups. They were housed individually, with food and water supplied *ad libitum*. The 'stressed group' was exposed to stress on PND 22, 23, and 24. The rats were killed and brain slices were prepared on PND 24 and 25. The experiments were performed in a blind manner. The investigators did not know whether they used a control or a stressed rat until the data were analyzed.

Stress exposure consisted of a 2-h per day session of immobilization and tail-shocks, for 3 consecutives days. The animals were stressed in the morning (between 0800 and 1200). They were restrained in a plexiglas tube, and 40 electric shocks (2 mA, 3 s duration) were applied at varying intervals (140–180 s). This stress protocol was adapted from the 'learned helplessness' paradigm in which animals undergo an aversive experience under conditions in which they cannot perform any adaptive response (Seligman and Maier, 1967; Seligman and Beagley, 1975). We stressed the rats for 3 consecutive days because it has been previously demonstrated that repeated stress sessions for 3 days is more effective than a single stress session in producing physiological and behavioral abnormalities, such as elevations in the basal plasma corticosterone levels, exaggerated acoustic startle responses, and reduced body weight (Servatius *et al*, 1995; Ottenweller *et al*, 1989). More stress sessions, beyond the 3 days, do not appear to produce greater physiological and behavioral changes (Servatius *et al*, 1995; Ottenweller *et al*, 1989).

Slice Preparation

Experimental procedures. The amygdala slice preparation has been described previously (Li *et al*, 2001). Briefly, the rats were anesthetized with halothane and then decapitated. The brain was rapidly removed and placed in an ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 11 glucose, bubbled with 95% O_2/5% CO_2. A block containing the amygdala region was prepared by rostral and caudal coronal cuts, and coronal slices, 400 μm thick, were cut using a Vibratome (series 1000, Technical Products International, St Louis, Missouri). Slices were kept in a holding chamber containing oxygenated ACSF at room temperature, and experiments started $\geqslant$1 h after slice preparation.

Electrophysiology

For whole-cell recordings, slices were transferred to a submersion-type recording chamber where they were continuously perfused with oxygenated ACSF at a rate of 4 ml/min. All experiments were carried out at 32°C. Tight-seal (>1 GΩ) whole-cell recordings were obtained from the cell body of neurons in the BLA region. Patch electrodes were fabricated from borosilicate glass and had a resistance of 1.5–5.0 MΩ when filled with a solution containing (in mM) Cs-gluconate, 135; $MgCl_2$, 10; $CaCl_2$, 0.1; EGTA, 1; HEPES, 10; QX-314, 20; NaATP, 2; Na_3GTP, 0.2 and Lucifer yellow, 0.4% (pH 7.3, 285–290 mOsm). Neurons were

visualized with an upright microscope (Nikon Eclipse E600fn) using the Nomarski-type differential interference optics through a $\times 60$ water immersion objective. Neurons with a pyramidal appearance were selected for recordings. During whole-cell recordings, neurons were filled passively with 0.4% Lucifer yellow (Molecular Probes, Eugene, Oregon) for *post hoc* morphological identification, as described previously (Braga *et al*, 2003). The fluorescence image of the dye-filled neurons was captured by a Leica DM RXA fluorescence microscope equipped with an SPOT2 digital camera and a laser scanning confocal microscope (Bio RAD, MRC-600). Neurons were voltage clamped using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Inhibitory postsynaptic currents (IPSCs) were pharmacologically isolated and recorded at a holding potential of -70 mV. Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes (2 μm diameter, World Precision Instruments, Sarasota, Florida) placed in the BLA, 50–100 μm from the recording electrode. Stimulation was applied, at 0.1 Hz, using a photoelectric stimulus isolation unit having a constant current output (PSIU6, Grass Instrument Co., W. Warwick, RI). Access resistance (8–26 MΩ) was regularly monitored during recordings, and cells were rejected if it changed by more than 15% during the experiment. The signals were filtered at 2 kHz, digitized (Digidata 1322A, Axon Instruments, Inc.), and stored on a computer using the pCLAMP8 software (Axon Instruments, Inc.). The peak amplitude, 10–90% rise time, and the decay time constant of IPSCs were analyzed off-line using pCLAMP8 software (Axon Instruments) and the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). Miniature IPSCs (mIPSCs) were analyzed off-line using the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ), and detected by manually setting the threshold for each mIPSC after visual inspection.

For field potential recordings, slices were transferred to an interface-type recording chamber maintained at 32°C, where they were perfused with ACSF at 0.7–1 ml/min. Field potentials were recorded in the BLA, while stimulation was applied to the external capsule, at 0.05 Hz (Aroniadou-Anderjaska *et al*, 2001). Recording glass pipettes were filled with 2 N NaCl (2–5 MΩ). Bipolar stimulating electrodes were constructed from twisted, stainless-steel wires, 50 μm in diameter. The field potentials were filtered at 1 kHz, and digitized on-line at 5 kHz.

All data are presented as mean ± SEM. For body weight data, sample size n refers to the number of rats. For electrophysiological experiments, sample size n refers to the number of slices. This corresponds to the number of neurons, in whole-cell recordings, as a single neuron was studied from each slice. From each rat, two slices were used for each type of experiment (whole-cell recordings or field potential recordings). The results were tested for statistical significance using the Student's paired t-test.

Drugs

The following drugs were used: D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5, Tocris Cookson, Ballwin, Missouri), an NMDA receptor antagonist; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Cookson, Ballwin, Missouri), a potent AMPA/kainate receptor antagonist; (2*S*)-(+)-5,5-dimethyl-2-morpholineacetic acid (SCH50911, Tocris Cookson, Ballwin, Missouri), a $GABA_B$ receptor antagonist; bicuculline methiodide (Sigma), a $GABA_A$ receptor antagonist; tetrodotoxin (TTX, Sigma), a sodium channel blocker; DL-propranolol (Sigma), a β adrenoceptor antagonist; (1-[4-amono-6,7-dimethoxy-2-quinazolinyl]-4-[2-furanylcarbonyl]-piperazine hydrochloride (prazosin, Sigma), an α_1 adrenoceptor antagonist; yohimbine hydrochloride (Sigma), an α_2 adrenoceptor antagonist; *N*-[5-(4,5-dihydro-1H-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydro-naphthalen-1-yl]methanesulfonamide hydrobromide (A61603, Tocris Cookson, Ballwin, Missouri), a selective α_{1A} agonist (Knepper *et al*, 1995); chloroethylclonidine (CEC, Sigma), an irreversible antagonist that blocks both α_{1B} and α_{1D} adrenoceptors (Xiao and Jeffries, 1998); 8-[2-[4-(2-methoxyphenyl)-1-piperazinil]ethyl]-8-azaspiro[4,5]-decane-7,9-dione dihydrochloride (BMY 7378, Tocris Cookson, Ballwin, Missouri), a selective antagonist of α_{1D} adrenoceptors (Deng *et al*, 1996; Saussy Jr *et al*, 1996); 2-(2,6-dimethoxyphenoxyethy)aminomethyl-1,4-benzodioxane hydrochloride (WB4101, Tocris Cookson, Ballwin, Missouri), a selective antagonist of the α_{1A} adrenoceptor (Zhong and Minneman, 1999).

RESULTS

The body weight of the control and stressed rats was measured daily between 1400 and 1500. The control rats were 44.5 ± 1.5 g ($n=24$) on PND 21 and 58.8 ± 1.9 g ($n=24$) on PND 24 (Figure 1). The body weight of the stressed group was 44.2 ± 1.8 g ($n=23$) before the first stress session on PND 21, and 51.0 ± 2.3 g ($n=20$) after the last stress session, on PND 24. The difference in body weight between stressed and control rats was statistically significant after the second day of stress ($p<0.01$). Stressed rats that were not used for electrophysiological experiments continued to display reduced body weight gain for as long as body weight was monitored (up to 10 days after stressor cessation, data not shown).

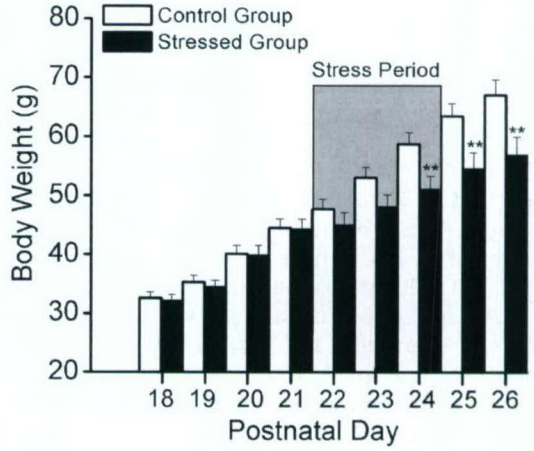


Figure 1 Restrain/tail-shock stress reduces body weight gain. Exposure to stress on PNDs 22, 23, and 24 reduced body weight gain. The body weight difference between control and stressed rats was statistically significant after the first day of stress (**$p<0.01$). Data on PND 26 are from rats that were not used for electrophysiological experiments. Sample sizes range from 12 (PND 26) to 24 rats.

Stress Blocks Noradrenergic Facilitation of GABAergic Synaptic Transmission

Noradrenergic modulation of spontaneous IPSCs (sIPSCs). To investigate whether NE modulates GABAergic transmission in the BLA, and whether stress alters this modulation, we first examined the effects of NE on action-potential dependent, sIPSCs recorded from BLA pyramidal neurons, in control and stressed rats. sIPSCs were recorded at a holding potential of −70 mV, and in the presence of D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM) to block NMDA, AMPA/kainate, and β and α_2 receptors, respectively. In control rats, the mean frequency of sIPSCs recorded from the soma of BLA pyramidal neurons was 3.1 ± 1.6 Hz ($n=21$). Bath application of bicuculline (10 μM) eliminated sIPSCs, confirming that they were mediated by $GABA_A$ receptors. NE at 1, 10, and 100 μM produced a dose-dependent enhancement in the frequency and amplitude of sIPSCs (Figure 2). At 100 μM of NE, the enhancement of sIPSCs was too high to be quantified precisely. The 10 μM concentration appeared to be close to the EC_{50}, and therefore it was used in subsequent experiments. After the application of 10 μM NE, the mean frequency of sIPSCs was increased to 984.39 ± 148.2% of the control values ($n=21$, $p<0.01$; Figure 3a). The amplitude of sIPSCs was increased to 144.0 ± 12.8% of the control values ($n=21$, $p<0.05$; Figure 3a). These effects persisted throughout the application of NE and were completely reversed after removal of the agonist. The effects of NE were not accompanied by any significant change in the rise time or decay time constant of sIPSCs (Figure 3a), and were blocked by the α_1 adrenoceptor antagonist prazosin (1 μM, Figure 3c), confirming that NE was acting via α_1 adrenergic receptors.

In stressed rats, the mean frequency of sIPSCs was 2.6 ± 2.3 Hz. NE (10 μM) had no significant effect on the frequency or amplitude of sIPSCs. Thus, in the presence of NE (10 μM), the frequency of sIPSCs was 128.9 ± 19.2% and the amplitude was 111.4 ± 10.2% of the control values ($n=19$, Figure 3b). In addition, bath perfusion of NE (10 μM) caused no significant changes in the kinetics of these currents (rise time and decay time constant of sIPSCs; Figure 3b).

To identify the subtype of α_1 adrenoceptors involved in the effects of NE on control rats, we first applied NE (10 μM) in the additional presence of CEC (10 μM) and BMY 7378 (300 nM) to block α_{1B} and α_{1D} adrenoceptors. There was no significant attenuation of the effects of NE in the presence of these antagonists (Figure 4). Thus, NE increased the frequency of sIPSCs from 2.8 ± 2.4 to 27.1 ± 7.9 Hz ($p<0.01$, $n=6$; Figure 4), and the amplitude of sIPSCs to 154 ± 11.3% of the control values ($p<0.05$, $n=6$; Figure 4).

Next, we examined the effects of the specific α_{1A} adrenoceptor agonist A61603. In control rats, A61603 (1 μM) increased the frequency and amplitude of sIPSC to 1034 ± 158.6 and 162 ± 14.2% of the control values, respectively ($p<0.01$, $n=16$; Figure 5a). There were no effects on the rise time or the decay time constant of sIPSCs (Figure 5a). In stressed rats, A61603 had no significant effect (Figure 5b). Thus, in the presence of 1 μM A61603 the frequency of sIPSCs was 132 ± 21% and the amplitude of sIPSCs was 106 ± 8.8% of the control values ($n=18$, Figure 5b). The effects of A61603 on sIPSCs in control rats were blocked by the selective α_{1A} adrenoceptor antagonist WB4101 (1 μM, Figures 5c and d).

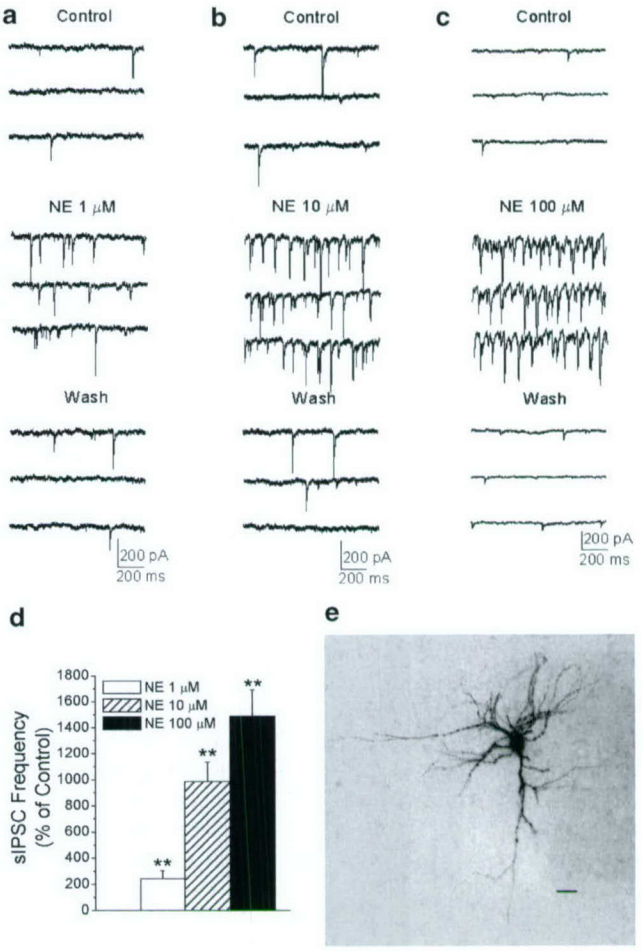
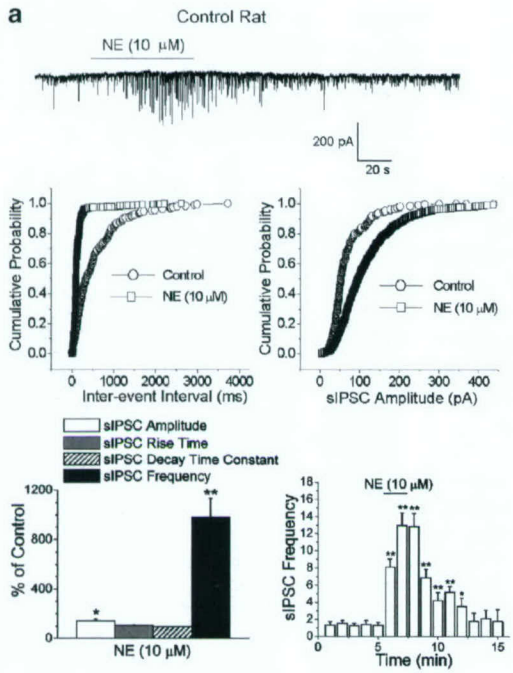
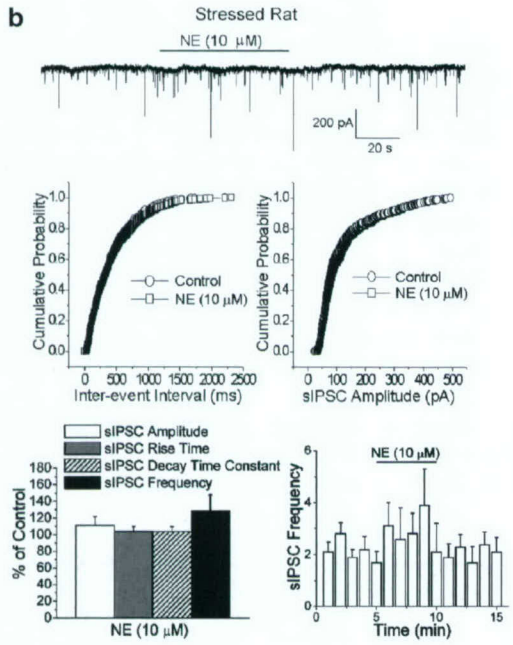


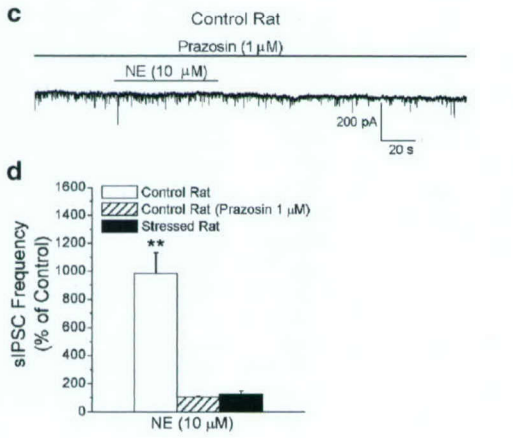
Figure 2 Activation of α_1 adrenoceptors increases tonic inhibition of BLA pyramidal neurons in a dose-dependent manner. (a–c) sIPSCs recorded from three different cells are shown. The holding potential is −70 mV. The medium contains D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM). The application of 1, 10, and 100 μM NE increased the frequency of sIPSCs in a dose-dependent manner. The bar graph (d) shows group data of the increase of sIPSC frequency ($n=8$ for each concentration of NE, $^{**}p<0.01$). (e) Photomicrograph of pyramidal cell (b) showing the typical morphology of the recorded neurons. The cell has been labeled with Lucifer Yellow. Scale bar, 40 μm.

Taken together, these results suggest that (1) NE, acting via α_{1A} adrenoceptors, enhances tonic inhibition of pyramidal cells in the BLA by inducing a massive increase in action potential-dependent spontaneous release of GABA, and (2) stress impairs this function of NE.

Noradrenergic modulation of evoked IPSCs (eIPSCs). It has been shown previously that NE reduces evoked inhibitory transmission in the hippocampus via α adrenoceptors (Madison and Nicoll, 1988; Doze *et al*, 1991). More recently, in the sensorimotor cortex, it was found that NE actually has a small facilitatory effect on eIPSCs, which is detected when $GABA_B$ receptors are blocked (Bennett *et al*, 1997). To determine the effects of NE on evoked inhibitory







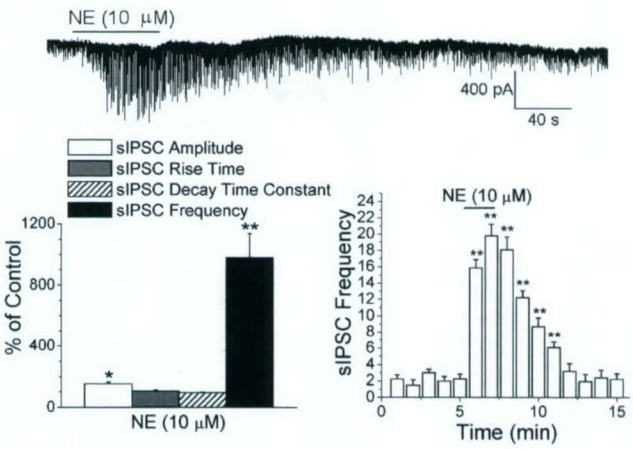
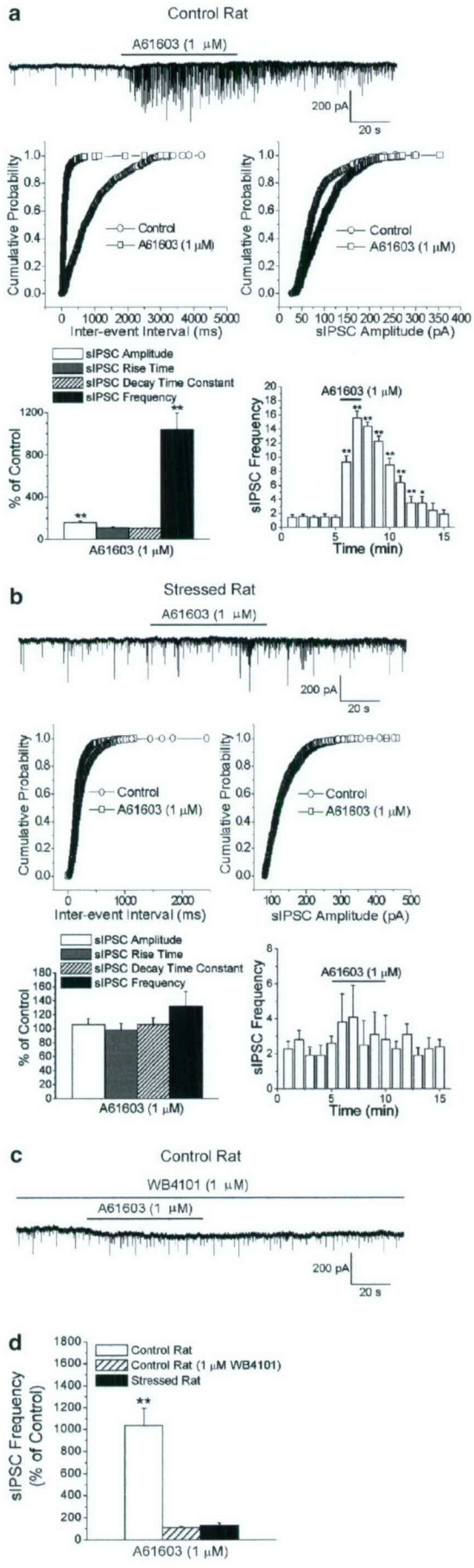


Figure 4 The NE-induced enhancement of sIPSCs is not blocked by α_{1B} and α_{1D} adrenoceptor antagonists. Top trace: sIPSCs recorded from a BLA pyramidal cell of a control rat (holding potential is −70 mV). Bath application of NE (10 μM) in the presence of D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), yohimbine (20 μM), CEC (10 μM), and BMY 7378 (300 nM) reversibly increased the frequency and amplitude of sIPSCs. The bar graph shows pooled data (mean ± SEM) from six neurons. $^*p<0.05$, $^{**}p<0.01$.

transmission in the BLA we applied 10 μM NE while recording eIPSCs in control rats. In the absence of a $GABA_B$ receptor antagonist, NE (10 μM) reduced the amplitude of eIPSCs to 48.2 ± 10.3% of the control levels ($p<0.01$, $n=8$; Figure 6). However, in the presence of SCH50911 (20 μM), a specific antagonist of the $GABA_B$ receptors, NE enhanced the amplitude of eIPSCs to 162.4 ± 9.3% of the control, $p<0.01$, $n=10$; Figure 7a) without affecting the rise time and decay time constant of the eIPSCs (Figure 7a). Similar effects were obtained when α_{1A} adrenoceptors were activated by the application of 1 μM A61603 (Figure 7c). Thus, A61603 (1 μM) increased the amplitude of eIPSCs to 159.4 ± 10.7% of the control ($p<0.01$, $n=8$, Figure 7c) without affecting the kinetics of the eIPSCs (Figure 7c). The effects of the drugs were

Figure 3 Activation of α_1 adrenoceptors increases tonic inhibition of BLA pyramidal neurons in control rats, but not in stressed rats. (a) Top trace: effects of NE (10 μM) on sIPSCs recorded from a BLA pyramidal cell of a control rat. The holding potential is −70 mV. The medium contains D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM). Middle graphs: cumulative probability plots of interevent intervals and amplitude of sIPSCs, in control conditions and during NE perfusion (same cell as in the top trace). Bottom graphs: pooled data (mean ± SEM) from 21 neurons. The bar graph on the left shows the NE-induced changes in amplitude, frequency, and kinetics of sIPSCs. The bar graph on the right panel shows the time course of changes in sIPSC frequency during the application of NE. $^*p<0.05$, $^{**}p<0.01$. (b) Top trace: sIPSCs recorded from a BLA pyramidal cell of a stressed rat (the holding potential is −70 mV); NE (10 μM) had no significant effect. Middle graphs: cumulative probability plots of interevent intervals and amplitude of sIPSCs in control conditions and during NE perfusion (same cell as in the top trace). Bottom graphs: pooled data (mean ± SEM) from 19 neurons. Effects of NE on the amplitude, kinetics, and frequency of sIPSCs in stressed rats. (c) Prazosin (1 μM) prevented the NE-induced increase of sIPSCs observed in control rats. (d) The bar graph shows the effects of NE on the mean frequency of sIPSCs recorded from control rats (in the absence and in the presence of prazosin), and stressed rats (in the absence of prazosin). $^*p<0.05$, $^{**}p<0.01$.



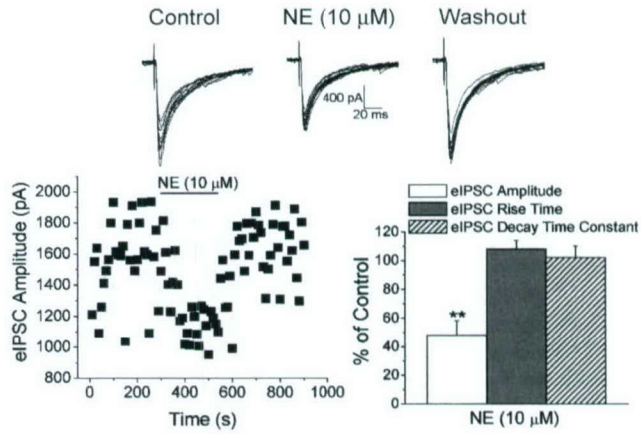


Figure 6 Activation of α_1 adrenoceptors reduces the amplitude of eIPSCs in control rats. Top traces: eIPSCs recorded from a BLA neuron of a control rat. The slice medium contains D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM). NE reduced the amplitude of eIPSCs with no significant effect on their kinetics. Bottom graphs: the plot shows the time course of the NE effects on the amplitude of eIPSCs (same cell as in top traces). The bar graph shows the relative (% of control) NE-induced changes in amplitude and kinetics of eIPSCs. Pooled data from eight neurons. $^{**}p<0.01$.

reversible. In stressed rats, neither NE nor A61603 had a significant effect on the amplitude, rise time, and decay time constant of eIPSCs (Figure 7b and d). In the presence of NE (10 μM), the eIPSC amplitude was 109 ± 8.2% of the control ($n=11$), and in the presence of A61603, the amplitude of the eIPSCs was 103 ± 7.4 % of the control ($n=10$). These results suggest that (1) NE facilitates evoked the GABAergic transmission via α_{1A} adrenergic receptors, (2) this facilitatory effect is masked due to the activation of presynaptic $GABA_B$ autoreceptors following the NE-induced enhancement of spontaneous GABA release, and (3) stress blocks the facilitatory effect of NE on evoked GABA release.

Noradrenergic modulation of mIPSCs. The enhancement of eIPSCs and action-potential-dependent sIPSCs by NE could be due to a depolarizing effect via the activation of

Figure 5 Activation of α_{1A} adrenoceptors increases tonic inhibition of BLA pyramidal neurons in control rats, but not in stressed rats. (a) Top trace: sIPSCs recorded from a BLA pyramidal cell of a control rat (the holding potential is −70 mV). Bath application of A61603 (1 μM), a specific α_{1A} adrenoreceptor agonist, reversibly increased the frequency and amplitude of sIPSCs. The slice medium contains D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM). Middle graphs: cumulative probability plots of sIPSC interevent intervals and amplitude in control conditions and during A61603 perfusion (same cell as in the top trace). Bottom graphs: bar graphs show pooled data (mean ± SEM) from 16 neurons. (b) sIPSCs recorded from a BLA pyramidal cell of a stressed rat (the holding potential is −70 mV). Bath application of A61603 (1 μM) caused no significant change in the frequency or amplitude of sIPSCs. Middle graphs: cumulative probability plots of sIPSCs interevent intervals and amplitude in control conditions and during A61603 (1 μM) perfusion (same cell as in the top trace). Bottom graphs: bar graph shows pooled data (mean ± SEM) from 18 neurons. (c) WB4101 (1 μM) prevented the A61603-induced effects observed in control rats. (d) Bar graph shows the effects of A61603 (1 μM) on the mean frequency of sIPSCs recorded from control rats (in the absence and in the presence of WB4101), and stressed rats (in the absence of WB4101). $^{*}p<0.05$, $^{**}p<0.01$.

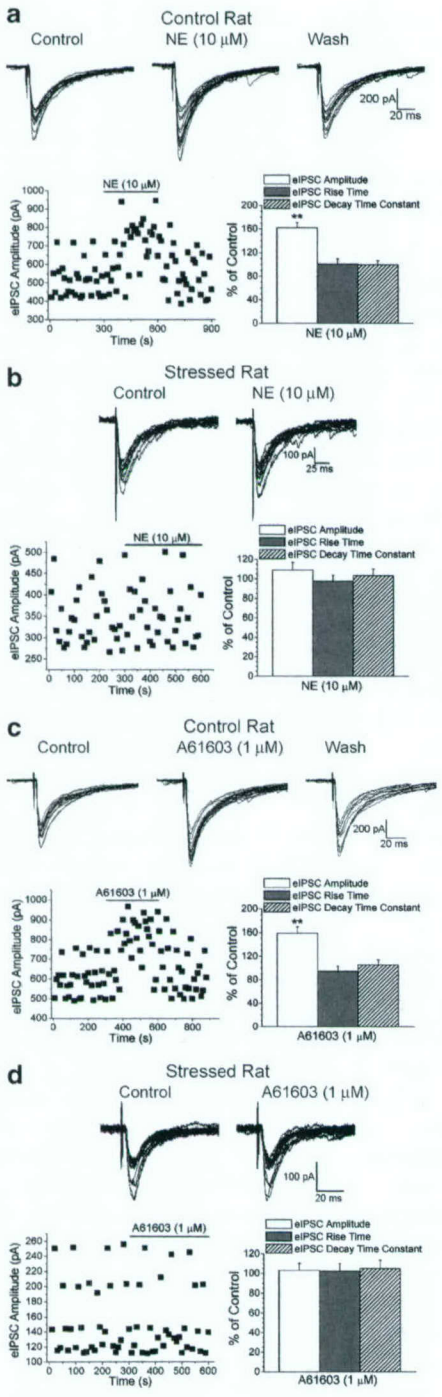


Figure 7 In the presence of a $GABA_B$ receptor antagonist, activation of α_{1A} adrenoceptors increases the amplitude of eIPSCs in control rats, but not in stressed rats. (a) Top traces: eIPSCs recorded from a BLA pyramidal cell of a control rat. In addition to D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM), the slice medium also contains 20 μM SCH50911. NE increased the amplitude of the eIPSCs, without affecting their kinetics. Bottom graphs: the plot shows the time course of the NE effect on eIPSC amplitude (same cell as in the top traces). The bar graph shows the effect of NE on the amplitude and kinetics of eIPSCs. Pooled data from 10 neurons. $^{**}p<0.01$. (b) Data similar to those shown in (a), but from stressed rats. The bar graph shows pooled data from 11 neurons. (c) In control rats, the α_{1A} agonist A61603 produced similar effects to those of NE. Top traces and bottom left plot show data from the same cell. The bar graph shows pooled data from eight BLA neurons. (d) In stressed rats, A61603 had no significant effects on eIPSCs. The bar graph shows pooled data from 10 BLA neurons.

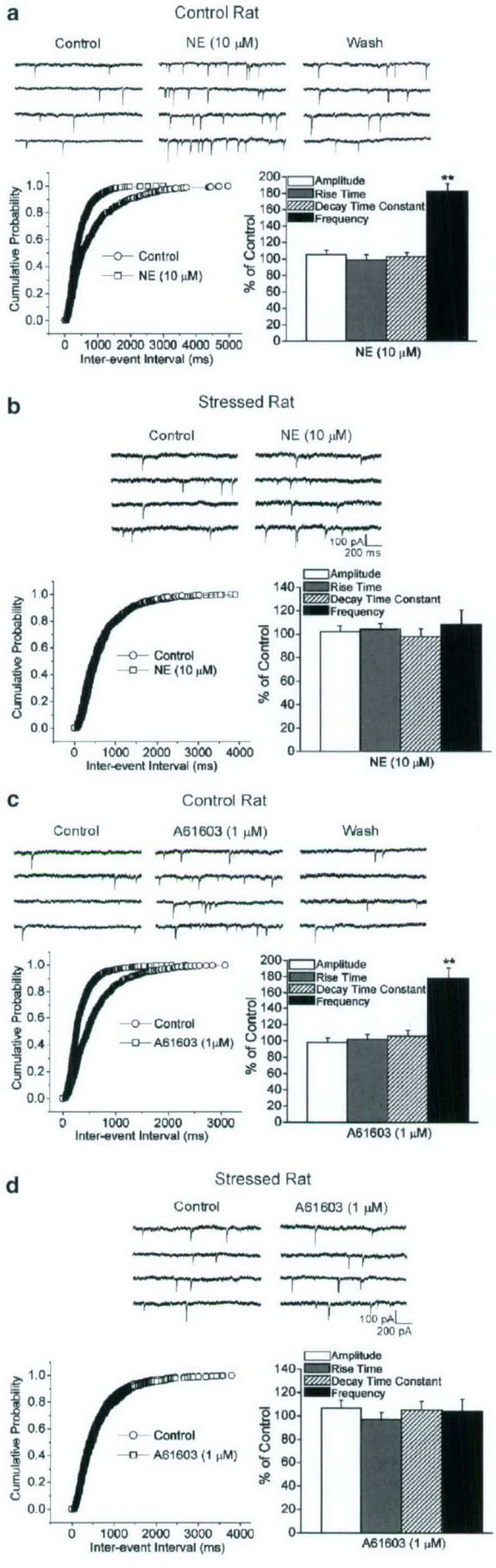
somatodendritic α_{1A} adrenoceptors on GABAergic neurons, and/or due to a direct effect at GABAergic terminals. To determine whether NE modulates GABA release by a direct effect on GABAergic terminals in the BLA, we tested the effects of NE on mIPSCs, which do not depend on the presynaptic invasion of action potentials or Ca^{2+} influx. mIPSCs were recorded in a medium containing D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), yohimbine (20 μM), and TTX (1 μM). In the absence of NE, the frequency of mIPSCs was 0.68 ± 0.32 Hz and their amplitude was 114.0 ± 12 pA ($n=10$). NE (10 μM) increased the frequency of mIPSCs to 182.3 ± 9.6 % of the control levels ($p<0.01$, $n=10$; Figure 8a). The amplitude, rise time, and decay time constant of the mIPSCs were not significantly affected by 10 μM NE (Figure 8a). Similar effects were observed after the application of the α_{1A}-specific agonist A61603 (Figure 8c). A61603 (1 μM) increased the frequency of mIPSCs from 0.71 ± 0.24 to 1.28 ± 0.31 (178 ± 12.4% of the control, $p<0.01$, $n=9$; Figure 8c). The amplitude and kinetics of mIPSCs were not affected by A61603 (Figure 8c).

In stressed rats, neither NE (10 μM) nor A61603 (1 μM) produced a significant effect on mIPSCs frequency, amplitude, or kinetics (Figure 8b and d). Thus, the frequency of mIPSCs was 0.68 ± 0.25 and 0.64 ± 0.34 Hz before and during the application of NE, respectively ($n=10$), and 0.72 ± 0.27 and 0.63 ± 0.31 Hz in the presence and absence of 1 μM A61603, respectively ($n=8$).

These results suggest that (1) NE facilitates GABA release by a direct effect on GABAergic terminals, and (2) this mechanism of noradrenergic facilitation of GABA release is impaired by stress.

Facilitation of GABAergic Transmission by α_{1A} Adrenoceptors is Mediated by Phospholipase C (PLC)

Studies in other brain regions or cell types have shown that α_1 adrenoceptors are coupled to PLC via a G-protein, and can increase the intracellular calcium concentration $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores, as well as by increasing the Ca^{2+} influx (Schwinn *et al*, 1991; Wu *et al*, 1992; Cohen and Almazan, 1993; Lepretre *et al*, 1994; Kulik *et al*, 1999). However, certain effects of α_{1A} activation involve signaling pathways that are independent of PLC activation and intracellular Ca^{2+} rise (Berts *et al*, 1999). To determine whether the α_{1A} adrenoceptor-mediated facilitation of GABA release, in the BLA, involves the activation of PLC, we examined whether the effects of NE on the GABAergic transmission are blocked by a PLC inhibitor. In control rats, NE (10 μM) or A61603 (1 μM) enhanced the frequency and amplitude of sIPSCs in the presence of U73343 (20 μM), the inactive isomer of the PLC inhibitor U73122, but had no effects in the presence of 20 μM U73122 (Figure 9). Thus, in the presence of U73343, NE increased the frequency of sIPSCs to 1022.8 ± 105.3% of the control levels ($p<0.01$, $n=8$; Figure 9a) and increased the amplitude of sIPSCs to 161 ± 11.7% ($p<0.01$, $n=6$; Figure 9a); A61603 (1 μM) increased the frequency of sIPSCs to 978.1 ± 102.1% ($p<0.01$, $n=8$; Figure 9b), and increased the amplitude of sIPSCs to 154 ± 12.3% of the control levels ($p<0.01$, $n=8$; Figure 9b). In contrast, in the presence of U73122 (20 μM), NE (10 μM) and A61603 (1 μM) failed to induce any significant changes in the frequency



and amplitude of sIPSCs (Figure 9c–e). Similarly, the effects of NE (10 μM) on the amplitude of eIPSCs, as well as on the frequency of mIPSCs, were blocked by 20 μM U73122 (not shown).

Stress Blocks α_{1A} Adrenoceptor-Mediated Suppression of BLA Field Potentials

Since the activation of α_{1A} adrenoceptors facilitates GABAergic transmission, the function of these receptors at the network level could be to dampen neuronal excitability and responsiveness. However, while spontaneous GABAergic activity is dramatically enhanced by activation of α_{1A} adrenoceptors (Figure 5), evoked GABAergic transmission is suppressed due to presynaptic inhibition of GABA release via $GABA_B$ autoreceptors (Figure 6). Therefore, under physiological conditions when $GABA_B$ receptors are not blocked, α_{1A} adrenoceptor activation could enhance the amygdala's responsiveness (due to the reduction in evoked GABA release), unless the enhancement of spontaneously released extracellular GABA plays a more decisive role in neuronal excitability. To determine the net effect of α_{1A} adrenoceptor activation on neuronal responsiveness and excitability in the BLA, and whether this effect is altered by stress, we investigated the effects of NE or A61603 on population, field responses, in the absence of $GABA_B$ receptor blockade, in control and stressed rats.

Field potentials in the BLA were evoked by stimulation of the external capsule. These responses consist of one major, negative component that corresponds in time course to the EPSP recorded intracellularly from BLA pyramidal cells (Aroniadou-Anderjaska *et al*, 2001; Chen *et al*, 2003), and is mediated by AMPA/kainate receptors (Aroniadou-Anderjaska *et al*, 2001). In control rats, 10 μM NE, in the presence of propranolol (10 μM) and yohimbine (20 μM), produced a significant reduction in the peak amplitude of evoked field potentials (83.8 ± 5.3% of control levels, $n = 14$, $p < 0.05$; Figure 10a). Similarly, bath application of 1 μM A61603 caused a significant reduction in the peak amplitude of the field potentials to 83.1 ± 5.2% of the control levels ($p < 0.05$, $n = 12$; Figure 10b). In contrast, in stressed rats, neither NE (10 μM) nor A61603 (1 μM) had a significant effect on the amplitude of the field potentials (Figure 10, bottom panels).

These results suggest that the function of α_{1A} adrenoceptors in the BLA is to reduce neuronal excitability/responsiveness, and this function is impaired by stress.

Figure 8 Activation of α_{1A} adrenoceptors increases the frequency of mIPSCs in control rats, but not in stressed rats. mIPSCs were recorded in the presence of TTX (1 μM), D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM). (a) Top traces: mIPSCs recorded from a BLA pyramidal neuron of a control rat. NE (10 μM) increased the frequency of mIPSCs. Bottom graph: the left panel shows the cumulative probability plots of interevent intervals of mIPSCs under control conditions and during the application of NE (same cell as in top traces). The bar graph shows the effect of NE on the amplitude, kinetics, and frequency of mIPSCs. Pooled data from 10 neurons, $^{**}p < 0.01$. (b) Similar data to those shown in (a), but from stressed rats. NE had no significant effect on mIPSCs. The bar graph shows pooled data from 10 neurons. (c) In control rats, the α_{1A} antagonist A61603 had similar effects to those induced by NE. The bar graph shows pooled data from nine BLA neurons. (d) A61603 had no significant effects on mIPSCs recorded from BLA pyramidal cells of stressed rats. The bar graph shows pooled data from eight cells.

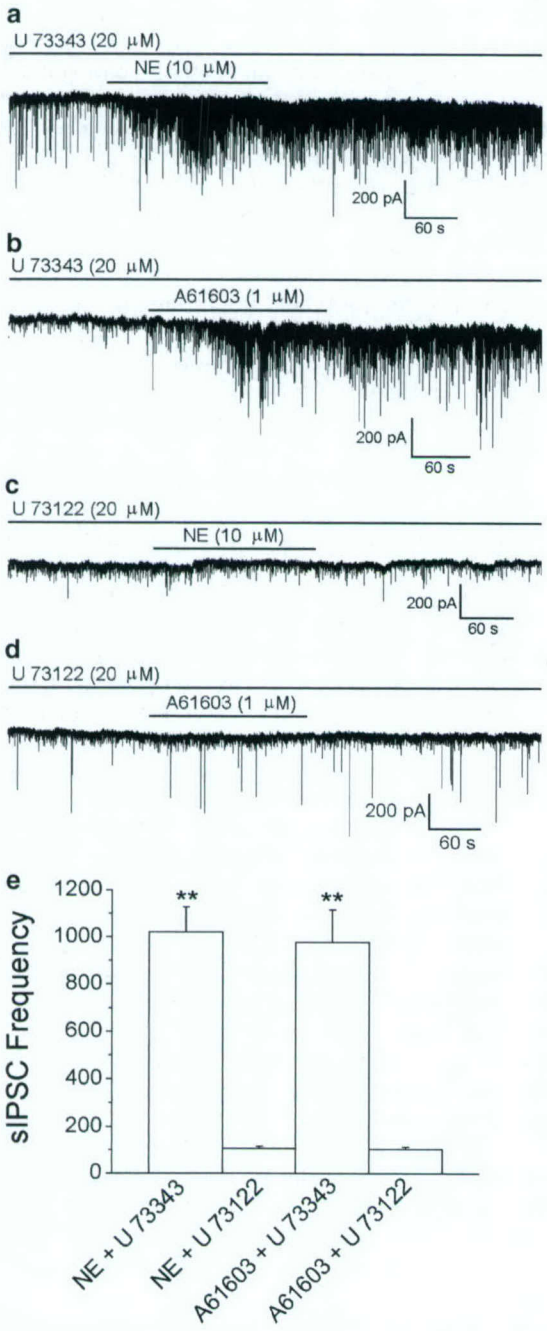


Figure 9 α_{1A} adrenoceptors in the BLA are coupled to PLC. (a–d) sIPSCs recorded from BLA pyramidal neurons. NE (a) or A61603 (b) increased the frequency and amplitude of sIPSCs in the presence of the inactive isomer of a PLC inhibitor (U73343), but had no effect in the presence of the PLC inhibitor U73122 (c and d). The slice medium contains D-AP5 (50 μM), CNQX (10 μM), propranolol (10 μM), and yohimbine (20 μM). (e) Bar graphs showing the effects of NE (10 μM) or A61603 (1 μM) on the frequency of sIPSCs in the presence of U73343 or U73122. Pooled data from eight neurons.

DISCUSSION

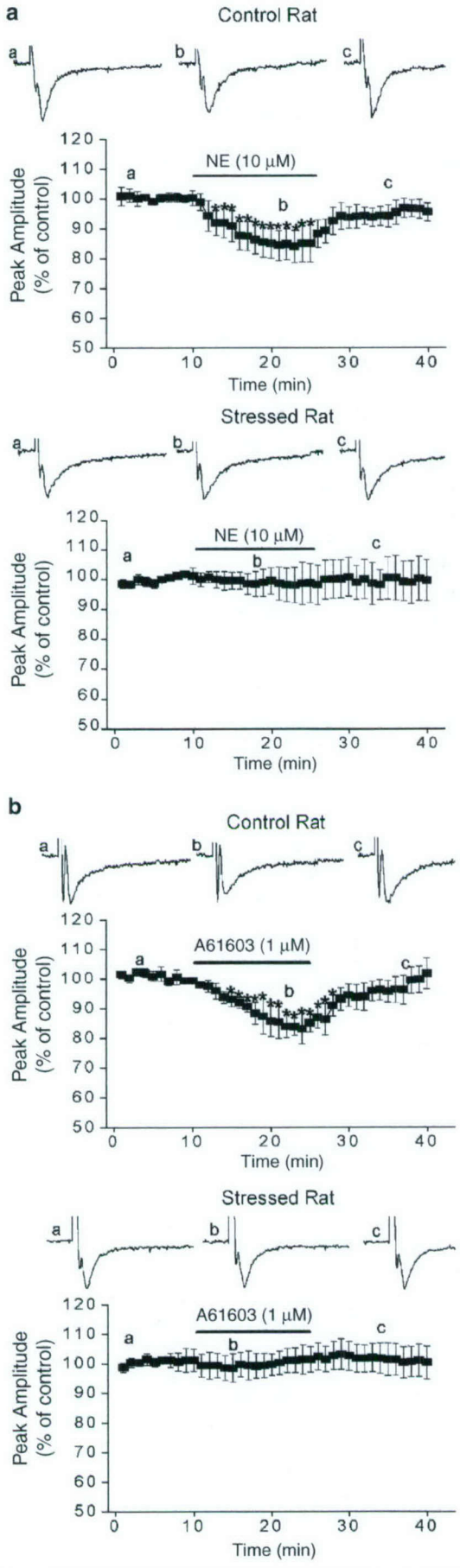
The present study describes two main findings. First, activation of the α_{1A} subtype of adrenergic receptors facilitates both tonic and phasic $GABA_A$ receptor-mediated inhibition of BLA pyramidal neurons. Second, stress produces a severe impairment of the α_{1A} adrenoceptor-mediated facilitation of GABAergic synaptic transmission in the BLA. These findings provide one possible explanation for (1) the antiepileptic effects of NE in temporal lobe epilepsy, (2) the amygdala's hyperexcitability in stress-related affective disorders, and (3) the stress-induced increase in the frequency of seizures in epileptic patients.

NE Facilitates GABAergic Transmission in the BLA via presynaptic α_{1A} Adrenoceptors

All three subtypes of α_1 adrenoceptors, α_{1A}, α_{1B}, and α_{1D}, are present in the amygdala, as determined by *in situ* hybridization (Day *et al*, 1997). The distribution of these receptors varies in different nuclei of the amygdala. The BLA expresses the α_{1A} adrenoceptor subtype almost exclusively (Day *et al*, 1997; Domyancic and Morilak, 1997). The role of these receptors in the amygdala's physiology and function has been unknown. In the present study, we show that NE, acting via the α_{1A} subtype of adrenergic receptors, facilitates GABA release in the BLA. Spontaneous, evoked, and quantal release of GABA were enhanced by NE or the specific α_{1A} adrenoceptor agonist A61603.

Endogenous NE released from noradrenergic terminals reaches its targets both by diffusion and via conventional synapses (Papadopoulos and Parnavelas, 1990; Seguela *et al*, 1990; Asan, 1993; Arce *et al*, 1994; Li *et al*, 2002). In the BLA, noradrenergic axons form asymmetric synapses with the dendrites of GABAergic neurons (Li *et al*, 2002). Although α_{1A} adrenoceptors may be located in such dendritic synapses and could be involved in the enhancement of spontaneous and evoked GABA release by NE, the increase in the frequency of mIPSCs by α_{1A} adrenoceptor activation indicates the presence of these receptors on GABAergic terminals. The enhancement of spontaneous GABA release by NE has also been observed in other brain regions (Madison and Nicoll, 1988; Doze *et al*, 1991; Gellman and Aghajanian, 1993; Alreja and Liu, 1996; Bergles *et al*, 1996; Bennett *et al*, 1997, 1998; Kawaguchi and Shindou, 1998), and it is mediated via α_1 adrenoceptors (Gellman and Aghajanian, 1993; Alreja and Liu, 1996; Bergles *et al*, 1996; Kawaguchi and Shindou, 1998); the specific α_1 receptor subtype involved has not been determined. At least in the CA1 hippocampal area, it appears that α_1 adrenoceptors are located only on somatodendritic regions of GABAergic cells, since mIPSCs are unaffected by adrenergic agonists (Bergles *et al*, 1996). Thus, the amygdala and the hippocampus may differ in the subcellular distribution of α_1 adrenoceptors mediating the facilitation of GABA release.

Evoked GABA release in the hippocampus is suppressed by NE, and this effect is also mediated via α adrenoceptors (Madison and Nicoll, 1988). However, a similar effect of NE in the sensorimotor cortex has been found to be due to the activation of presynaptic $GABA_B$ autoreceptors; when $GABA_B$ receptors were blocked, NE enhanced evoked GABAergic transmission (Bennett *et al*, 1997). Similarly, in the present study, the facilitatory effect of NE on evoked GABAergic transmission was revealed only when $GABA_B$ receptors were blocked, suggesting that the accumulation of extracellular GABA due to the NE-induced enhancement of spontaneous GABA release inhibited evoked GABA release.



Since NE enhances spontaneous GABA release, but suppresses evoked GABA release when $GABA_B$ receptors are functional, this raises the question of what would be the net effect of α_{1A} adrenoceptor activation on the overall excitability and responsiveness of the amygdala. The BLA field potentials were reduced by NE or A61603 in the absence of $GABA_B$ receptor antagonists. It is unlikely that this effect is due to a reduction in glutamate release, because glutamatergic transmission in the BLA is suppressed via α_2, but not α_1 adrenoceptor activation (Ferry *et al*, 1997). Thus, the reduction of the BLA field potentials by NE or A61603 suggests that the dramatic enhancement of spontaneously released GABA induced by α_{1A} adrenoceptor activation (Figure 5) over-rides the reduction in evoked GABAergic transmission (Figure 7) producing a suppression of the amygdala's excitability.

The intracellular signaling mechanisms that mediate the physiological effects of α_{1A} adrenoceptor activation in the BLA involve the activation of PLC, since a PLC inhibitor prevented the enhancement of sIPSCs, eIPSCs, and mIPSCs by NE and A61603. The activation of PLC may lead to mobilization of Ca^{2+} from intracellular stores, and/or Ca^{2+} influx, following phosphoinositide hydrolysis and formation of IP3, as it has been observed in different tissues and cell types following α_1 adrenoceptor activation (Schoepp and Rutledge, 1985; Schwinn *et al*, 1991; Perez *et al*, 1993; Kulik *et al*, 1999; Zhong and Minneman, 1999; Khorchid *et al*, 2002), or α_{1A} adrenoreceptor activation (Cohen and Almazan, 1993; Lepretre *et al*, 1994). In the present study, since NE or A61603 enhanced the frequency of mIPSCs, the influx of Ca^{2+} through voltage-gated calcium channels is not necessary for the α_{1A} adrenoreceptor-mediated facilitation of GABA release in the BLA.

The amygdala is a key player in the pathogenesis and symptomatology of temporal lobe epilepsy (Gloor, 1992; Weiss *et al*, 2000; Avoli *et al*, 2002). NE has long been known to display anticonvulsant properties, but little is known about the underlying mechanisms (Chen *et al*, 1954; Stanton, 1992; Stanton *et al*, 1992; Szot *et al*, 1999; Stoop *et al*, 2000; Weinshenker *et al*, 2001) The α_{1A} adrenoreceptor-mediated facilitation of GABA release in the BLA may be one of the mechanisms involved in the antiepileptic effects of NE in temporal lobe seizure disorders.

Stress Impairs the Function of α_{1A} Adrenoceptors in the BLA

Previous studies have suggested that excessive or repeated stress can produce long-lasting alterations in the amygdala's structure and function. Thus, chronic immobilization, in rats, induces hypertrophy of the dendritic arborizations of

Figure 10 Activation of α_{1A} adrenoceptors reduces BLA field potentials in control rats, but not in stressed rats. (a) Changes in the peak amplitude of BLA field potentials evoked by stimulation of the external capsule, in response to bath application of 10 M NE, in control (top panel, $n = 9$) and stressed (bottom panel, $n = 10$) rats. The medium contains propranolol (10 M) and yohimbine (20 M). (b) Similar data to those in (a), except that A61603 is applied in place of the NE. Pooled data from 10 slices (control rats, top panel) and eight slices (stressed rats, bottom panel). The slice medium same as in (a). Asterisks over error bars denote statistically significant reduction ($p < 0.05$).

pyramidal and stellate neurons in the BLA (Vyas *et al*, 2002; Pawlak *et al*, 2003). Fear conditioning or other types of stressors such as exposure to a predator produce long-lasting changes in the efficacy of synaptic transmission in the amygdala (LeDoux, 1992; Davis *et al*, 1994; Rogan *et al*, 1997, McKernan and Shinnick-Gallagher, 1997; Adamec *et al*, 2001). In human patients with stress-related affective disorders, the amygdala exhibits hypertrophy (Strakowski *et al*, 1999; Altshuler *et al*, 2000), increased levels of basal activity (Drevets, 1999), or exaggerated responses to fearful stimuli (Rauch *et al*, 2000). In the present study, repeated restrain/tail-shock stress produced a severe impairment in the α_{1A} adrenoreceptor-mediated facilitation of GABA release in the BLA, indicating that stress impairs the function of α_{1A} adrenoceptors. This impairment could result from receptor desensitization, internalization, or down-regulation, or by an effect on the intracellular signaling pathways activated by PLC. In other brain regions, repeated stress reduces mRNA levels of α_1 adrenoceptors (Miyahara *et al*, 1999). Adrenergic receptors desensitize or undergo downregulation following prolonged exposure to the agonist (Yang *et al*, 1999; Chalothorn *et al*, 2002). Thus, during stress exposure, excessive release of NE in the amygdala (Galvez *et al*, 1996; Quirarte *et al*, 1998; Tanaka *et al*, 2000) may be responsible for the impairment of the α_{1A} adrenoreceptor function. In addition, previous studies have shown that restrain/tail-shock stress elevates plasma corticosterone levels (Servatius *et al*, 1995). Glucocorticoid receptors colocalize with α_1 adrenoceptors (Fuxe *et al*, 1985; Williams *et al*, 1997), and it has been demonstrated that corticosterone downregulates α adrenoceptors (Stone *et al*, 1986, 1987; Joels and de Kloet, 1989). Therefore, another possibility is that the corticosterone released during exposure to stress downregulates α_{1A} adrenoceptors. An important question is whether the impairment in the α_{1A} adrenoceptor function is a transient or a long-term effect. The investigations described here focus on changes measured within a relatively short period of time after stressor cessation. However, preliminary experiments have revealed differences in the α_{1A} adrenoceptor function between stressed and control rats on the fifth day after the termination of stress exposure, suggesting that the stress-induced dysfunction in the noradrenergic modulation of GABA release is not likely to be a short-term effect.

Functional implications. What are the possible functional implications of a stress-induced loss of the α_{1A} adrenoceptor-mediated noradrenergic facilitation of GABA release in the BLA? In the normal amygdala, basal levels of NE, acting via α_{1A} adrenoceptors, may contribute to tonic inhibition of BLA pyramidal neurons, by facilitating both action potential-dependent and -independent GABA release. The loss or impairment of this facilitation would result in hyperexcitability at rest, and a lower threshold of activation. When the normal amygdala is activated in response to an emotionally significant event triggering the release of NE, activation of α_{1A} adrenoceptors will facilitate the role of inhibitory transmission in active neuronal circuits; this role is not only to prevent overexcitation, but also to shape and sharpen the flow of excitatory activity. Therefore, loss of the α_{1A} adrenoceptor-mediated facilitation of synaptic inhibition may result in inappropriate overactivation of the amygdala and impairment in the processing and interpretation of an emotional stimulus. A dysfunction of this nature may also affect the formation of emotional memories. In the normal amygdala, noradrenergic facilitation of GABAergic transmission may either suppress memory formation (due to the suppression of excitation), or facilitate optimal registration of the memory trace (by regulating the level and flow of excitatory activity). In a hyper-responsive amygdala, when noradrenergic facilitation of GABA release is impaired, events of little emotional significance may be registered as significant, and memories of emotionally significant events may be 'overconsolidated'. It should be noted, however, that the net effect of stress on the function of the noradrenergic system in the BLA remains to be determined, as stress may also induce changes in the interaction of NE with other adrenoceptor subtypes (β and α_2) or neurotransmitter systems.

It has been hypothesized that the hyperactivity and hyper-responsiveness of the amygdala associated with certain affective disorders, such as PTSD, is due to the loss of proper cortical modulation of the amygdala, and/or due to an intrinsic lower threshold of amygdala response to emotionally significant stimuli (Villarreal and King, 2001). The present findings suggest that a reduction in GABAergic transmission due to the loss of the α_{1A} adrenoceptor-mediated facilitation of GABA release may be one of the mechanisms responsible for the apparently reduced threshold of amygdala's activation in these affective disorders. The present findings also suggest that a stress-induced impairment in the function of α_{1A} adrenoceptors, which could result in reduced tonic inhibition in the BLA, may be one of the mechanisms underlying the stress-induced increased frequency of seizures in patients with temporal lobe epilepsy (Temkin and Davis, 1984; Frucht *et al*, 2000). Moreover, our results suggest that the reduced central α_1 adrenoceptor responsiveness (Asnis *et al*, 1985, 1992), and binding (Crow *et al*, 1984; Gross-Isseroff *et al*, 1990) in depressed patients may be stress-related, and that one of the physiological consequences of this reduction is an impaired modulation of the GABAergic transmission.

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Bidirectional Modulation of GABA Release by Presynaptic Glutamate Receptor 5 Kainate Receptors in the Basolateral Amygdala

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The activation of kainate receptors modulates GABAergic synaptic transmission, but the mechanisms are currently a matter of intense debate. In the basolateral amygdala (BLA), the glutamate receptor 5 (GluR5) subunit of kainate receptors is heavily expressed, and GluR5 antagonists block a novel form of synaptic plasticity; yet little is known about the role of GluR5-containing kainate receptors in the physiology of the amygdala. Here we show that GluR5 agonists bidirectionally modulate the strength of synaptic transmission from GABAergic interneurons to pyramidal cells in a concentration-dependent manner. Low concentrations of (*RS*)-*S*-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) (ATPA) (0.3 μM) or glutamate (5 μM) reduced the number of failures of GABAergic synaptic transmission and enhanced the frequency of miniature IPSCs (mIPSCs). High concentrations of ATPA (10 μM) or glutamate (200 μM) increased the number of synaptic failures and reduced the frequency of mIPSCs. The facilitation or suppression of GABAergic transmission by the GluR5 agonists did not require activation of voltage-gated calcium channels or presynaptic $GABA_B$ receptors. It was also found that extracellular, endogenous glutamate tonically reduces the rate of failures of GABAergic transmission. These results suggest that the terminals of GABAergic neurons in the BLA carry two subtypes of GluR5-containing kainate receptors, which have different agonist affinities and activate opposing mechanisms of action. The GluR5-mediated, bidirectional modulation of GABA release by glutamate in the BLA may play an important role in the regulation of synaptic plasticity and neuronal excitability in this structure, under normal and pathological conditions.

Key words: GluR5; presynaptic kainate receptors; glutamate diffusion; GABA release; inhibitory synaptic transmission; amygdala

Introduction

Glutamate, the principal excitatory neurotransmitter in the vertebrate CNS, mediates fast synaptic transmission through three classes of ionotropic receptors: NMDA, AMPA, and kainate receptors. Although the roles of NMDA and AMPA receptors on synaptic transmission and plasticity have been studied extensively, the functions of kainate receptors have only recently begun to be unveiled. In addition to mediating excitatory synaptic transmission in some brain regions (Castillo et al., 1997; Vignes and Collingridge, 1997; Cossart et al., 1998; Li and Rogawski, 1998; Kidd and Isaac, 1999), kainate receptors have been shown to modulate the release of glutamate and GABA (for review, see Frerking and Nicoll, 2000; Kullmann, 2001). The modulation of GABA release by kainate receptors has been studied primarily in the hippocampus, where significant discrepancies have become apparent with regard to the nature of this regulation. A consistent finding is that kainate receptor activation enhances action potential-dependent, spontaneous GABA release (Cossart et al., 1998; Frerking et al., 1998; Mulle et al., 2000; Rodriguez-Moreno et al., 2000; Schmitz et al., 2000; Jiang et al., 2001; Semyanov and Kullmann, 2001). However, there is intense controversy with regard to the effects of kainate on evoked- or action potential-independent GABA release, the latter revealed by recording miniature IPSCs (mIPSCs) in the presence of TTX. Thus, in interneuron to CA1 pyramidal-cell synapses, depression (Rodriguez-Moreno et al., 1997) or no effect (Frerking et al., 1999; Cossart et al., 2001) of kainate on the frequency of mIPSCs has been reported. In the same type of synapses, most investigators have observed the depression of evoked GABAergic synaptic transmission after the application of kainate (Clarke et al., 1997; Rodriguez-Moreno et al., 1997, 2000; Frerking et al., 1999; Mulle et al., 2000), whereas others have seen both facilitation and depression, depending on the concentration of kainate (Jiang et al., 2001). The facilitation of GABAergic transmission by kainate has also been observed in hypothalamic neurons (Liu et al., 1999), whereas both facilitation and depression, depending on the concentration of kainate, were observed in dorsal horn neurons (Kerchner et al., 2001). The factors contributing to these discrepancies are unclear. The concentration of kainate seems to play an important role in the direction of the modulation of GABA release (Jiang et al., 2001; Kerchner et al., 2001), although this does not appear to hold true for interneuron-to-interneuron synapses, where an increase in mIPSC frequency has been observed with either low (Cossart et al., 2001) or high (Mulle et al., 2000) kainate concentrations. Thus, the type of synapses investigated may also play a role, particularly because an increase in the frequency of mIPSCs by kainate has been observed in interneuron-to-interneuron synapses (Mulle et al., 2000; Cossart et al., 2001) but not in interneuron-to-pyramidal-cell synapses (Rodriguez-Moreno et al., 1997; Frerking et al., 1999; Cossart et al., 2001). Yet

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others have seen no effect of kainate on mIPSCs recorded from interneurons (Frerking et al., 1998; Semyanov and Kullmann, 2001).

Considerable effort has also been made to determine the sites of action of kainate receptor agonists, but the results have not been conclusive (Kullmann, 2001). Thus, the facilitation of GABAergic transmission has been attributed to a direct effect of kainate at the presynaptic terminals of interneurons (Mulle et al., 2000; Cossart et al., 2001) and/or to depolarization of interneurons via axonal and somatodendritic kainate receptors (Semyanov and Kullmann, 2001). The depression of GABAergic transmission has also been attributed to a direct presynaptic effect of kainate (Rodriguez-Moreno and Lerma, 1998; Rodriguez-Moreno et al., 2000). However, others have argued that the kainate-induced depression of evoked GABAergic transmission is secondary to a dramatic enhancement in spontaneous GABA release induced by kainate, with accumulated GABA acting presynaptically on $GABA_B$ inhibitory receptors (Frerking et al., 1999; Kerchner et al., 2001) and/or postsynaptically by desensitizing $GABA_A$ receptors or by decreasing pyramidal-cell input resistance, thereby shunting evoked IPSCs (eIPSCs) (Frerking et al., 1999).

One difficulty in reconciling the results from different laboratories and reaching definitive conclusions is that there are different subtypes of kainate receptors that may be located in different types of cells or neuronal compartments and that may serve different functions (Chittajallu et al., 1999). In many studies, the subtype of receptors activated by kainate is not identified, because specific agonists/antagonists are currently available only for the glutamate receptor 5 (GluR5) subtype. When the GluR5 kainate receptor has been investigated, the specific GluR5 agonist (*RS*)-*S*-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) (ATPA) (Clarke et al., 1997) has been found to enhance action potential-dependent, spontaneous GABA release (Cossart et al., 1998; Rodriguez-Moreno et al., 2000), whereas it suppresses evoked GABA release (Clarke et al., 1997; Rodriguez-Moreno et al., 2000) in interneuron-to-pyramidal-cell synapses in the CA1 hippocampal area. ATPA also depresses eIPSCs recorded from neocortical pyramidal cells (Ali et al., 2001) but has virtually no effect in dorsal horn neurons (Kerchner et al., 2001). In interneuron-to-interneuron synapses, ATPA has no effect on mIPSC frequency (Cossart et al., 2001). The results reported by Mulle et al. (2000) in GluR5-deficient mice are consistent with the lack of involvement of the GluR5 subunit in the regulation of GABA release in interneuron-to-interneuron synapses, whereas Mulle et al. (2000) suggest that in interneuron-to-pyramidal-cell synapses, the GluR5 subunit together with the GluR6 subunit mediates suppression of evoked GABA release.

In the basolateral amygdala (BLA), the GluR5 subunit is highly expressed, and GluR5-containing kainate receptors seem to play a significant role, because they participate in synaptic transmission (Li and Rogawski, 1998) as well as in synaptic plasticity (Li et al., 1998, 2001). However, the precise location and function of GluR5 kainate receptors are unknown. In this study, we applied the whole-cell patch-clamp technique to investigate whether GluR5 kainate receptors modulate the GABAergic inhibition of identified pyramidal neurons in the basolateral amygdala.

Materials and Methods

Electrophysiology. Coronal slices containing the amygdala were prepared from 15- to 22-d-old male Sprague Dawley rats. The rats were anesthetized with halothane and then decapitated. The brain was rapidly removed and placed in ice-cold artificial CSF (ACSF) composed of (in mM): 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 11 glucose, bubbled with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. A block containing the amygdala region was prepared by rostral and caudal coronal cuts, and 400-μm-thick slices were cut using a Vibratome (series 1000; Technical Products International, St. Louis, MO). Slices were kept in a holding chamber containing oxygenated ACSF at room temperature, and recordings were initiated $\geq$1 hr after slice preparation.

For whole-cell recordings, slices were transferred to a submersion-type recording chamber, where they were continuously perfused with oxygenated ACSF at a rate of 3–4 ml/min. Neurons were visualized with an upright microscope (Nikon Eclipse E600fn; Nikon, Tokyo, Japan) using Nomarski-type differential interference optics through a 60× water immersion objective. All experiments were performed at room temperature (28°C). Tight-seal (>1 GΩ) whole-cell recordings were obtained from the cell body of neurons in the BLA region. Patch electrodes were fabricated from borosilicate glass and had a resistance of 1.5–5.0 MΩ when filled with a solution containing (in mM): 135 Cs-gluconate, 10 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, 10 HEPES, 2 Na-ATP, 0.2 Na_3GTP, and 0.4% Lucifer yellow, pH 7.3 (285–290 mOsm). Neurons were voltage-clamped using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). IPSCs were pharmacologically isolated and recorded at the reversal potential for glutamatergic events (V_{hold}, +10 mV). Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes (2 μm diameter; World Precision Instruments, Sarasota, FL) placed in the BLA, 50 μm from the recording electrode. Minimal stimulation was applied at 0.1 Hz, using a photoelectric stimulus isolation unit with a constant current output (PSIU6; Grass Instruments, West Warwick, RI).

Stimulation of a single presynaptic axon was confirmed by the intensity-threshold test (Raastad et al., 1992; Isaac et al., 1996; Bolshakov et al., 1997). The mean IPSC amplitude showed a steep all-or-none threshold as a function of stimulating current intensity. Increasing current intensity by 40–60% above the threshold for evoking an IPSC had no effect on the IPSC amplitude, indicating the stimulation of a single presynaptic axon. Access resistance (5–24 MΩ) was regularly monitored during recordings, and cells were rejected if it changed by >15% during the experiment. The signals were filtered at 2 kHz, digitized (Digidata 1322A; Axon Instruments), and stored on a computer using pClamp8 software (Axon Instruments). The peak amplitude, 10–90% rise time, and decay time constant of IPSCs were analyzed off-line using pClamp8 software and the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). The visual method was used to identify failures and to calculate failure rates (Stevens and Wang, 1994). The percentage of failures was calculated from at least 30 stimulus pulses before, during, and after agonist application. mIPSCs were analyzed off-line using the Mini Analysis Program and detected by manually setting the mIPSC threshold (~1.5 times the baseline noise amplitude) after visual inspection. In some experiments, cadmium was used to block voltage-gated calcium channels. Complete blockade of eIPSCs in the presence of 100 μM cadmium was used as a positive control for the efficacy of cadmium to block presynaptic calcium channels. In another series of experiments, postsynaptic currents were elicited by AMPA application. The fast application of AMPA was performed with a picospritzer (General Valve, Fairfield, NJ) at 3 min intervals. The pressure varied from 60 to 80 psi.

All data are presented as means ± SEM. The results were tested for statistical significance using the Student's paired *t* test.

Drugs. The following drugs were used: D-APV (Tocris Cookson, Ballwin, MO); an NMDA receptor antagonist, (+)-(2*S*)-5,5-dimethyl-2-morpholineacetic acid (SCH50911; Tocris Cookson); a $GABA_B$ receptor antagonist, bicuculline methiodide (Sigma, St. Louis, MO); a $GABA_A$ receptor antagonist, ATPA (Sigma); a GluR5 agonist (Clarke et al., 1997); and TTX (Sigma), a sodium channel blocker. The active isomer of GYKI 53655, an AMPA receptor antagonist, and the GluR5 antagonist (3*SR*,4a*RS*,6*RS*,8a*RS*)-6-[2-(iH-tetrazol-5-yl)ethyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroiso-quinoline-3-carboxylic acid (LY293558; Bleakman et al., 1996) were gifts from Lilly Research Laboratories (Eli Lilly and Co., Indianapolis, IN).

Morphology. During whole-cell recordings, neurons were filled passively with 0.4% Lucifer yellow (Molecular Probes, Eugene, OR) for *post hoc* morphological identification. Slices were fixed for 1 hr at 4°C in PBS, pH 7.4, containing 4% paraformaldehyde; they were subsequently mounted and coverslipped in 10% PBS in glycerine. The fluorescence image of the dye-filled neurons was captured by a Leica (Nussloch, Germany) DM RXA fluorescence microscope equipped with a SPOT2 digital camera and a laser scanning confocal microscope (MRC-600; Bio-Rad, Hercules, CA).

Protein solubilization and immunoblotting of GluR5 protein. For protein solubilization, the tissue was sonicated and subsequently solubilized in lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin). Cell lysates were rotated end-over-end at 4°C for 60 min, and insoluble material was pelleted at 12,000 × *g* for 30 min at 4°C. The protein concentrations of clarified tissue lysates were determined by the simplified Bradford method (Bio-Rad). The proteins were analyzed by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a transfer unit (Multiphor Novablot; Amersham Biosciences, Clayton, NC). After transfer, blots were incubated in the blocking buffer (0.02 M Tris-HCl, pH 7.6, 0.137 M NaCl, 1% bovine serum albumin) before blotting with the first antibody. Anti-GluR5 antibody (1:1000) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-actin antibody (1:200) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The first antibodies were incubated overnight at 4°C. The blots were washed in washing buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 0.25% Tween 20) and then incubated with horseradish peroxidase-conjugated secondary antibodies (Transduction Laboratories, Lexington, KY). Enhanced chemiluminescence substrate (Amersham Biosciences, Piscataway, NJ) was used according to the manufacturer's instructions for antibody detection and exposed to x-ray film.

Results

Activation of GluR5 kainate receptors bidirectionally modulates the failure rate of GABAergic synaptic transmission

To determine whether GluR5 kainate receptors modulate action potential-dependent GABA release from BLA interneurons, we investigated the effects of ATPA (0.3–10 μM), a selective agonist for GluR5 kainate receptors (Clarke et al., 1997), on the probability of failure of GABAergic synaptic transmission evoked by electrical activation of a presynaptic axon. eIPSCs were recorded from BLA pyramidal cells at a holding potential of +10 mV, and in the presence of D-APV (50 μM), SCH50911 (20 μM), and GYKI 53655 (50 μM). As shown previously in other brain areas (Wilding and Huettner, 1995; Rammes et al., 1996), 50 μM GYKI 53655 was sufficient to completely block AMPA receptor-mediated currents in the BLA (Fig. 1*a*), and therefore the recorded eIPSCs were not contaminated by AMPA currents. In addition, the eIPSCs were blocked by 10 μM bicuculline (Fig. 1*b*), which confirms that they were mediated by $GABA_A$ receptors. Application of ATPA at a 300 nM concentration decreased the number of failures of eIPSCs from 46.2 ± 8.1 to 12.4 ± 6.4% ($n = 9$; $p < 0.01$) (Fig. 2*a*). At 1 μM, ATPA caused a transient reduction in the number of failures, followed by a long-lasting increase in the failure rate, from 43.4 ± 7.9 to 65.5 ± 9.1% ($n = 10$; $p < 0.05$) (Fig. 2*b*). At a concentration of 10 μM, ATPA caused a marked increase in the rate of failures of eIPSCs from 49.2 ± 9.8 to 92.0 ± 5.1% ($n = 8$; $p < 0.01$) (Fig. 2*c*), which in most cells was apparent within 1 min of application of the agonist. Perfusion of the slices with ATPA-free ACSF completely reversed the effects of the agonist (Fig. 2). In addition, coapplication of ATPA (0.3–10 μM) with the selective GluR5 kainate receptor antagonist LY293558 (30 μM) had no significant effect on the number of failures of eIPSCs (59.8 ± 9.3% in 300 nM ATPA; 62.7 ± 5.9% in 1 μM ATPA; 67.1 ± 8.2% in 10 μM ATPA; $n = 9$; $p > 0.1$) compared with control (62.8 ± 6.2%) (Fig. 3*a*).

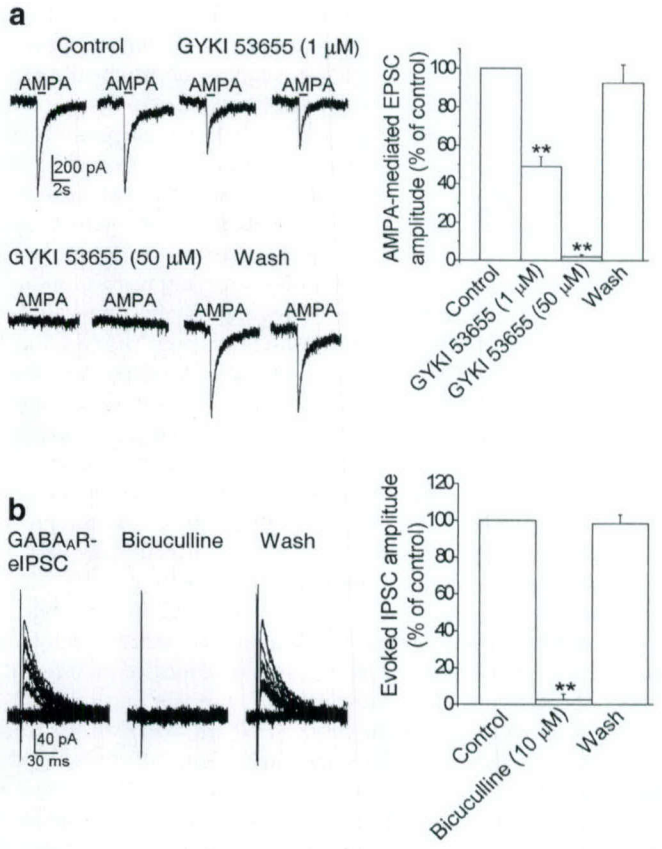

Figure 1. Postsynaptic currents evoked in the presence of 50 μM GYKI 53655, 50 μM D-APV, and 20 μM SCH59011 are $GABA_A$ receptor-mediated IPSCs. *a*, GYKI 53655, at a concentration of 50 μM, completely blocks AMPA-evoked postsynaptic currents. Sample traces of postsynaptic currents evoked by puff application of 200 μM AMPA in the presence of 1 μM TTX, 50 μM bicuculline, 50 μM D-APV, and 20 μM SCH59011 (holding potential, −70 mV) are shown. These currents were reduced by 52.1 ± 5.2% ($n = 8$) in the presence of 1 μM GYKI 53655 and were reversibly eliminated by 50 μM GYKI 53655. The *horizontal bar* above the current traces denotes the duration of the puff. The graph shows pooled data from eight neurons. $^{**}p < 0.01$. *b*, Postsynaptic currents recorded in the presence of 50 μM GYKI 53655, 50 μM D-APV, and 20 μM SCH59011 were blocked by 10 μM bicuculline. Superimposed traces are evoked currents recorded from a BLA pyramidal neuron (holding potential, +10 mV). The graph shows pooled data (means ± SEM) from nine neurons. $^{**}p < 0.01$.

To investigate whether the effects of ATPA could be mimicked by the endogenous agonist, we tested whether glutamate was capable of inducing similar changes on the rate of failures of evoked inhibitory synaptic transmission. In the presence of GYKI 53655 (50 μM), D-APV (50 μM), SCH50911 (20 μM), and 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt; 30 μM) to block AMPA, NMDA, $GABA_B$, and group I metabotropic GluR receptors, respectively, glutamate (5–200 μM) also produced a dose-dependent, bidirectional effect on evoked inhibitory synaptic transmission. A reduction in the number of failures of eIPSCs was observed at 5 μM glutamate, from a 37.0 ± 10.4 to a 9.1 ± 7.2% failure rate ($n = 6$; $p < 0.01$) (Fig. 4*a*), and enhancement was seen at higher concentrations; thus, the failure rate was increased from 52.4 ± 8.8 to 74.3 ± 7.1% with 30 μM glutamate ($n = 6$; $p < 0.01$) (Fig. 4*b*) and from 42.2 ± 10.3 to 96.9 ± 3.9% with 200 μM glutamate ($n = 5$;

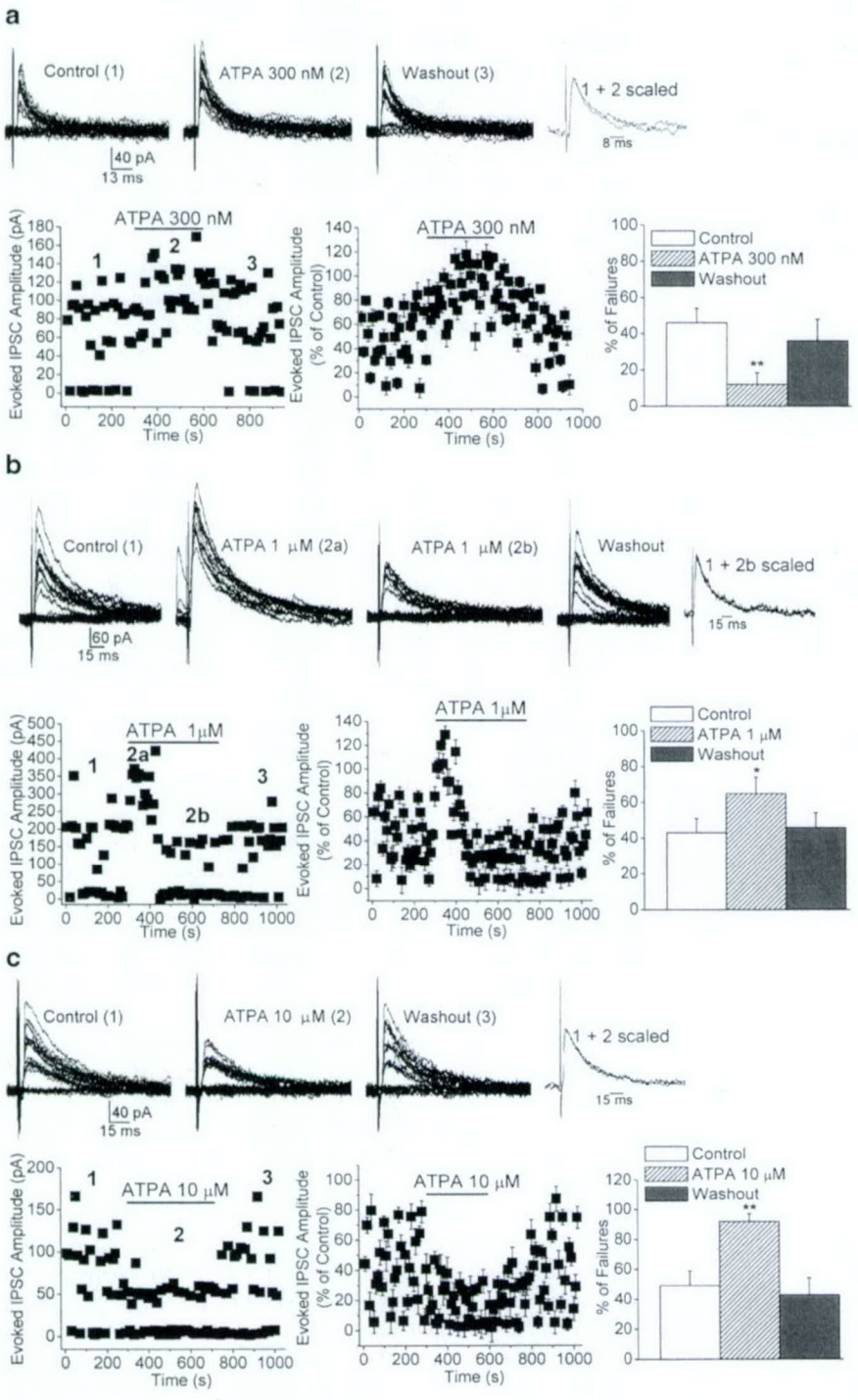

Figure 2. Dose-dependent, bidirectional modulation of the rate of failures of evoked GABAergic synaptic transmission by the GluR5 agonist ATPA. *a–c*, Superimposed traces are eIPSCs recorded from three different BLA pyramidal neurons before, during, and after bath application of different concentrations of ATPA. The scaled superimposed traces show that the effects of ATPA were not accompanied by changes in the kinetics of the eIPSCs. The plots show the time course of the effects of ATPA on the amplitude and number of failures of eIPSCs. Bar graphs are pooled data (means ± SEM) of the percentage of failures before, during, and after the application of ATPA. eIPSCs were recorded in the presence of GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM), at a holding potential of +10 mV. *a*, At 300 nM, ATPA significantly decreased the percentage of failures of eIPSCs ($n = 9$; $^{**}p < 0.01$). *b*, At 1 μM, ATPA caused a transient reduction (*2a*) followed by a long-lasting increase (*2b*) in the number of failures ($n = 10$; $^{*}p < 0.05$). *c*, At 10 μM, ATPA produced a marked increase in the percentage of failures of eIPSCs ($n = 8$; $^{**}p < 0.01$). Perfusion of the slices with ATPA-free ACSF completely reversed the effects of the agonist.

$p < 0.01$) (Fig. 4*c*). LY293558 completely prevented the effects of glutamate (5–200 μM). In the presence of LY293558 (30 μM), the failure rate was 61.9 ± 7.4% in 5 μM glutamate, 60.5 ± 6.7% in 30 μM glutamate, and 64.5 ± 7.3% in 200 μM glutamate ($n = 6$; $p > 0.1$) compared with control levels (63.1 ± 5.2%) (Fig. 3*b*).

Excitation of BLA interneurons via activation of GluR5 kainate receptors

The effects of ATPA on the failure rate of GABAergic transmission could be explained by a presynaptic action of the GluR5 agonist at GABAergic terminals. However, previous work in the hippocampus has shown that kainate depolarizes GABAergic interneurons via somatodendritic (Cossart et al., 1998; Frerking et al., 1998) and axonal (Semyanov and Kullmann, 2001) kainate receptors. If similar effects are induced on BLA interneurons by GluR5 kainate receptor activation, these effects could be responsible at least for the observed reduction in the number of failures of GABAergic transmission. Indeed, when AMPA, NMDA, $GABA_A$, and $GABA_B$ receptors were blocked by GYKI 53655 (50 μM), D-APV (50 μM), bicuculline (10 μM), and SCH50911 (20 μM), respectively, EPSCs evoked by electrical stimulation of the external capsule (3 shocks delivered at 100 Hz, every 10 sec) were recorded from BLA interneurons (Fig. 5*a*). These evoked EPSCs were completely blocked by bath application of LY293558 (30 μM) (Fig. 5*a*). Thus, GluR5 kainate receptors are present on the somatodendritic regions of BLA interneurons and mediate a component of the evoked EPSCs. Furthermore, application of ATPA (0.3–10 μM) in the presence of GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM) increased the frequency of action potential-dependent spontaneous IPSCs (sIPSCs) recorded in the soma of BLA pyramidal neurons (to 244.5 ± 61.3% by 300 nM ATPA, 572.4 ± 49.1% by 1 μM ATPA, and 908.2 ± 138.6% by 10 μM ATPA; $n = 9$; $p < 0.01$) (Fig. 5*b*). The effect persisted throughout the application of ATPA, was fully reversed after washout of the agonist, and was blocked by coapplication of LY293558 (30 μM) (Fig. 5*b*). The ATPA-induced increase in sIPSC frequency could be mimicked by glutamate. The application of glutamate (5–200 μM) in the presence of GYKI 53655 (50 μM), D-APV (50 μM), SCH50911 (20 μM), and CPCCOEt (30 μM) also increased the frequency of IPSCs recorded in the soma of BLA pyramidal neurons (to 303.8 ± 101.7% by 5 μM glutamate, 588.1 ± 112.3% by 30 μM glutamate, and 867.8 ± 144.1% by 200 μM glutamate; $n = 6$; $p < 0.01$). The glutamate-induced increase in sIPSC frequency was completely blocked by LY293558 (30 μM). Bath application of LY293558 (30 μM) alone did not affect the frequency of sIPSCs (5.8 ± 1.2 and

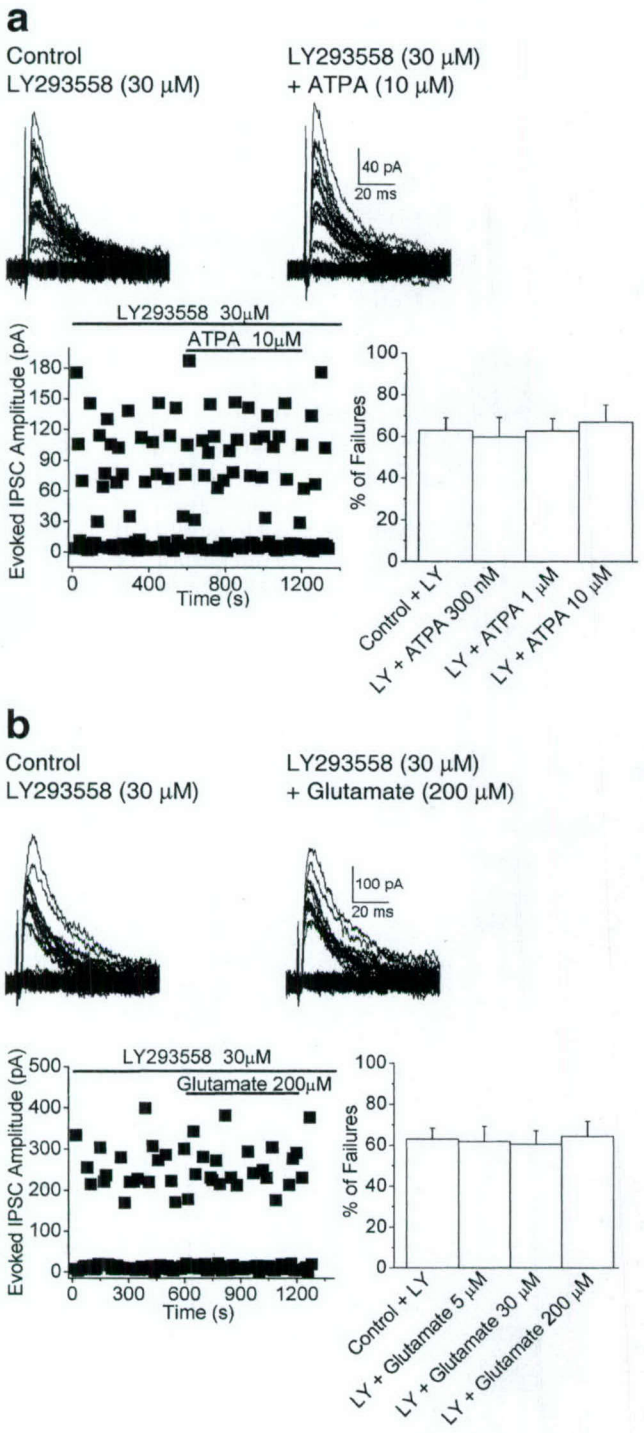

Figure 3. The effects of ATPA and glutamate on the rate of failures of GABAergic synaptic transmission are blocked by LY293558 (30 μM). *a*, *b*, Superimposed traces are eIPSCs recorded from pyramidal neurons in the presence of LY293558. Application of ATPA or glutamate had no effect on the failure rate of the eIPSCs. The plots show the amplitude of the eIPSCs versus time, before and during application of ATPA (*a*) or glutamate (*b*). Bar graphs are pooled data (means ± SEM) of the percentage of failures in the presence or absence of the GluR5 agonists. eIPSCs were recorded in the presence of GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM), at a holding potential of +10 mV.

6.2 ± 1.4 Hz in the absence and in the presence of LY293558, respectively; $n = 8$; $p > 0.1$), suggesting that GluR5 kainate receptors on somatodendritic and/or axonal regions of BLA interneurons are not tonically activated by endogenous glutamate.

Activation of GluR5 kainate receptors bidirectionally modulates the frequency of mIPSCs

Because ATPA and glutamate can depolarize BLA interneurons and induce spontaneous firing (Fig. 5), the reduction in the rate of failures of eIPSCs by low concentrations of ATPA (300 nM) or glutamate (5 μM) could be attributable to the activation of GluR5 kainate receptors located on somatodendritic and/or axonal regions of the interneurons, rather than to direct effects on GABAergic terminals. Such depolarization of interneurons by ATPA could produce either more glutamate release from the stimulated axon or the recruitment of an additional axon (Semyanov and Kullmann, 2001). However, the activation of GluR5 kainate receptors by higher concentrations of ATPA (1–10 μM) or glutamate (30–200 μM), which also caused the depolarization of BLA interneurons and therefore might be expected to reduce the number of failures of eIPSCs, induced a marked increase in the rate of failures (Figs. 2*b*,*c*, 3*b*,*c*, respectively). In other CNS areas, it has been proposed that the depression of GABAergic transmission by kainate is attributable to the extracellular accumulation of spontaneously released GABA, which activates presynaptic $GABA_B$ autoreceptors and/or desensitizes postsynaptic $GABA_A$ receptors (Frerking et al., 1999; Frerking and Nicoll, 2000; Kerchner et al., 2001). However, in our studies the blockade of $GABA_B$ receptors by SCH50911 did not prevent the inhibition of evoked GABA release by high concentrations of ATPA or glutamate. In addition, there were no signs of significant desensitization of $GABA_A$ receptors during application of either ATPA or glutamate, as determined by the kinetics of the currents (rise time and decay time constant of eIPSCs) (Figs. 2, 3). Accumulated extracellular GABA may also decrease pyramidal-cell input resistance, increasing the shunting of eIPSCs during electrotonic propagation from the synapse to the pyramidal cell soma, as suggested in the hippocampus (Frerking et al., 1999). In the present study, input resistance was measured from the responses to ±10 pA, 200 msec pulses applied at the resting membrane potential of pyramidal cells. Before the application of ATPA, the mean input resistance was 340 ± 18 MΩ ($n = 7$). ATPA, at 10 μM, caused a reduction in the input resistance of BLA pyramidal cells (to 44 ± 9% of control; $n = 5$; $p < 0.01$), but no clear correlation between the changes in the passive membrane properties and a reduction in eIPSC amplitude was observed. More importantly, 1 μM ATPA, which significantly enhanced the frequency of sIPSCs and increased the number of failures of eIPSCs, had no significant effect on the input resistance of BLA pyramidal cells (348 ± 24 MΩ in the presence of 1 μM ATPA; $n = 7$; $p > 0.1$). Therefore, it seems unlikely that a reduction in the input resistance of pyramidal neurons is responsible for the depression of eIPSCs by high concentrations of ATPA or glutamate.

To determine whether ATPA and glutamate acted directly on GABAergic terminals to modulate inhibitory transmission, we examined the effects of these agonists on mIPSCs, recorded in the presence of TTX (1 μM), GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM). A change in the frequency of mIPSCs without a change in their amplitude is indicative of a change in the probability of quantal release at the presynaptic terminals. The mean frequency of mIPSCs recorded in the soma of BLA pyramidal neurons was 3.7 ± 0.8 Hz ($n = 25$). Bath application of bicuculline (10 μM) eliminated mIPSCs, confirming that these were GABAergic currents. ATPA, at 300 nM, caused a significant increase in the mean mIPSC frequency (138.9 ± 9.3% of the control values; $n = 9$; $p < 0.01$) (Fig. 6*a*) that persisted throughout application of ATPA and was completely reversed after removal of the agonist. These effects of ATPA were not accompanied by any significant change in the amplitude, rise time, or

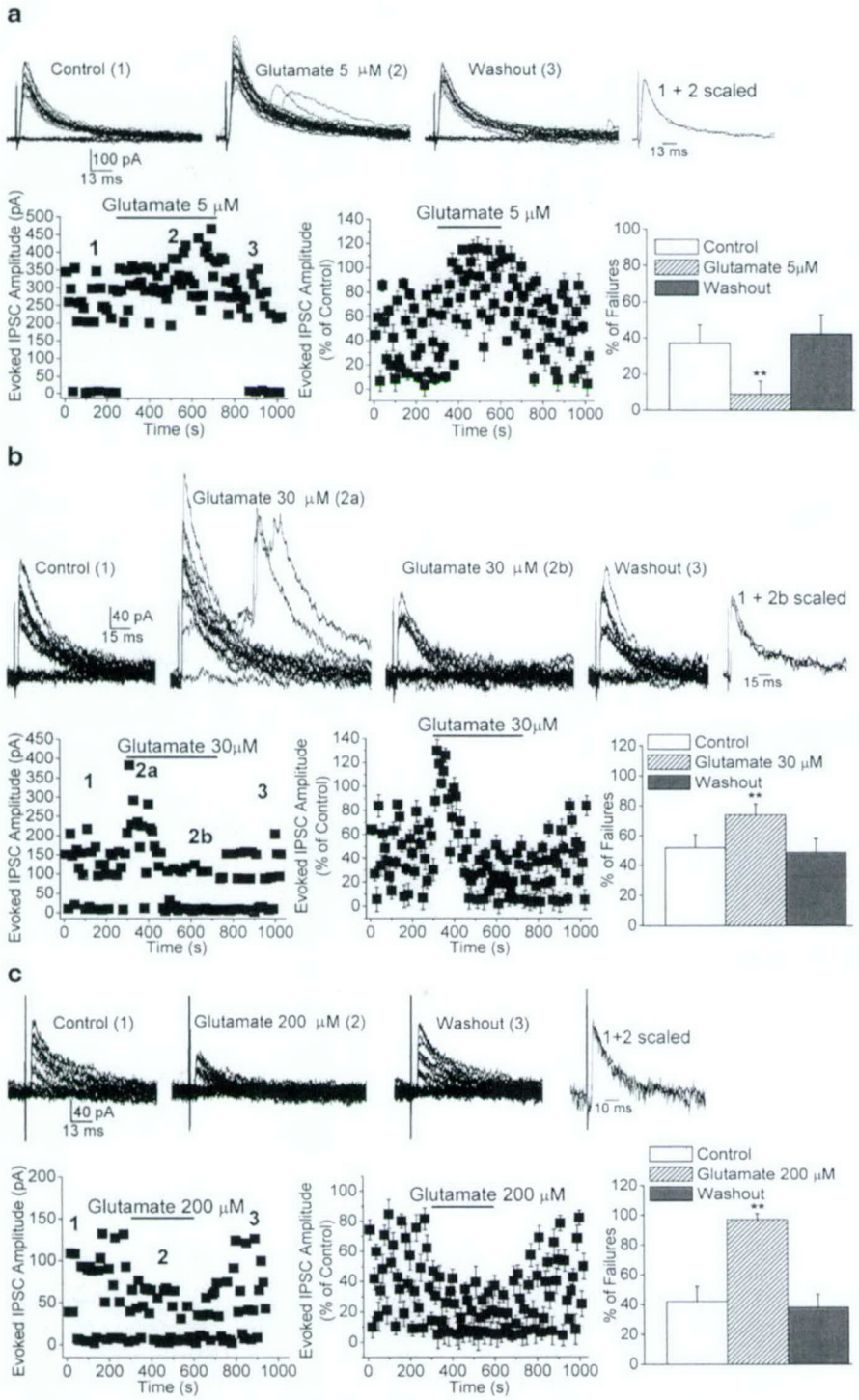

Figure 4. Dose-dependent, bidirectional modulation of the rate of failures of evoked GABAergic synaptic transmission by glutamate, via activation of GluR5 kainate receptors. *a–c*, Superimposed traces are eIPSCs recorded from three different BLA pyramidal neurons before, during, and after the application of glutamate. The scaled superimposed traces show that the effects of glutamate were not accompanied by changes in the kinetics of the eIPSCs. The plots show the time course of the effects of glutamate on the amplitude and number of failures of eIPSCs. Bar graphs are pooled data (means ± SEM) of the percentage of failures before, during, and after the application of glutamate. eIPSCs were recorded in the presence of GYKI 53655 (50 μM), D-APV (50 μM), SCH50911 (20 μM), and CPCCOEt (30 μM), at a holding potential of +10 mV. *a*, At 5 μM, glutamate decreased the percentage of failures of eIPSCs ($n = 6$; $^{**}p < 0.01$). *b*, At 30 μM, glutamate caused a transient reduction (*2a*) followed by a long-lasting increase (*2b*) in the number of failures ($n = 6$; $^{**}p < 0.01$). *c*, At 200 μM, glutamate caused a marked increase in the percentage of failures of eIPSCs ($n = 5$; $^{**}p < 0.01$). Perfusion of the slices with glutamate-free ACSF completely reversed the effects of the agonist.

decay time constant of mIPSCs (Fig. 6*a*). At 1 μM, ATPA reduced the mean frequency of mIPSCs to 74.8 ± 8.6% of control values ($n = 7$; $p < 0.05$) (Fig. 3*b*), and at 10 μM, the mean frequency of mIPSCs was further reduced to 64.0 ± 9.4% ($n = 7$; $p < 0.01$) (Fig. 6*c*). The reduction in the frequency of mIPSCs persisted throughout application of ATPA (1 or 10 μM) and was completely reversed after washout of the agonist. No significant changes in the amplitude, rise time, or decay time constant of mIPSCs were observed (Fig. 6*b*,*c*). Furthermore, bath application of LY293558 (30 μM) completely prevented the effects on the mean frequency of mIPSCs caused by ATPA (104.3 ± 7.6% of control in 300 nM ATPA, 96.2 ± 10.1% in 1 μM ATPA, and 93.5 ± 9.7% in 10 μM ATPA; $n = 4–7$; $p > 0.1$).

Next, we tested whether the dose-dependent, bidirectional effects of ATPA on mIPSC frequency could be mimicked by the endogenous agonist glutamate. We found that in the presence of TTX (1 μM), GYKI 53655 (50 μM), D-APV (50 μM), SCH50911 (20 μM), and CPCCOEt (30 μM), glutamate (5 μM) caused a significant increase in the mean mIPSC frequency (162.9 ± 11.7% of control values; $n = 3$; $p < 0.05$). In contrast, at a concentration of 200 μM, glutamate reduced the mean frequency of mIPSCs to 61.7 ± 9.3% of control values ($n = 3$; $p < 0.05$).

The effects of ATPA or glutamate on the frequency of mIPSCs provide strong evidence for the presence of GluR5 kainate receptors on GABAergic terminals of BLA interneurons. However, it is conceivable that even in the presence of TTX, the activation of GluR5 kainate receptors located on somatodendritic and/or axonal compartments of GABAergic interneurons could induce sufficient passive depolarization of nerve terminals, activating voltage-sensitive calcium channels, calcium entry, and vesicle fusion. Thus, to test whether the enhanced rate of mIPSCs could be mediated by calcium influx through voltage-sensitive calcium channels, we recorded mIPSCs in the presence of cadmium (100 μM), GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM). Application of ATPA (300 nM) caused a significant increase in the mean mIPSC frequency (147 ± 11.1%; $n = 6$; $p < 0.05$) (Fig. 7*a*) that was not significantly different from that in the absence of cadmium. Similarly, the presence of cadmium (100 μM) did not affect the reduction in the frequency of mIPSCs induced by 10 μM ATPA (55.8 ± 12.6%; $n = 3$; $p < 0.05$) (Fig. 7*b*). Thus, the effect of ATPA on the frequency of mIPSCs is mediated by GluR5 kainate receptors located on presynaptic GABAergic terminals and does not require Ca^{2+} influx through voltage-sensitive calcium channels.

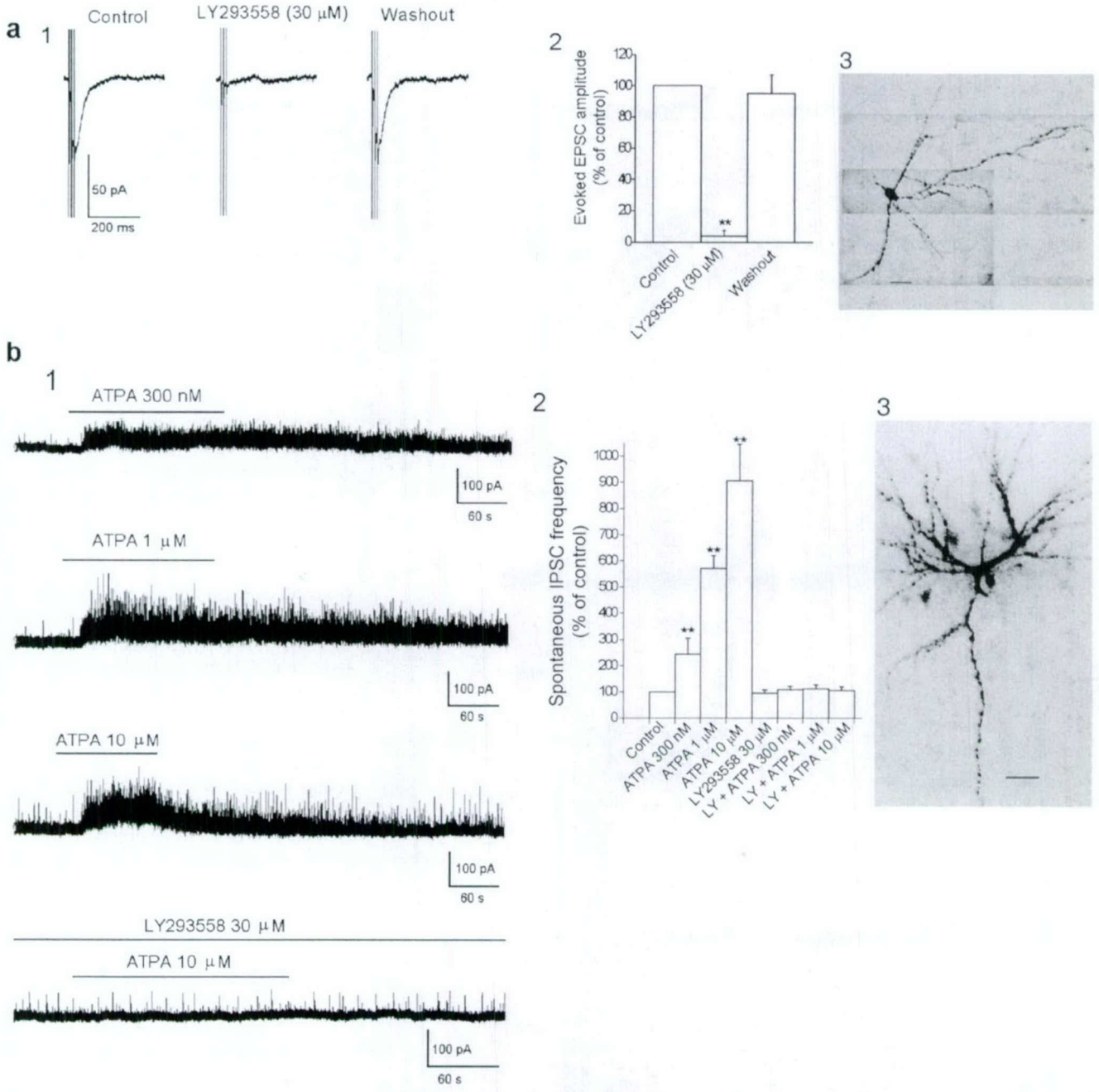

Figure 5. Excitation of BLA interneurons via GluR5 kainate receptors. *a*, Kainate receptors mediate a component of the synaptic responses of BLA interneurons. *1*, EPSCs (V_{hold}, −60 mV) recorded from a BLA interneuron in the presence of GYKI 53655 (50 μM), D-APV (50 μM), bicuculline (10 μM), and SCH50911 (20 μM). Electrical stimulation was applied to the external capsule (3 shocks delivered at 100 Hz, every 10 sec). *2*, Pooled data (means ± SEM) illustrating the blockade of evoked EPSCs, recorded as in *1*, by bath application of 30 μM LY293558 ($n = 8$; $^{**}p < 0.01$). A photomicrograph of the interneuron recorded in *a1* is shown in *3*. Scale bar, 50 μm. *b*, Activation of GluR5 kainate receptors increases the spontaneous activity of BLA interneurons. *1*, Effects of different concentrations of ATPA on sIPSCs recorded from the soma of three different BLA pyramidal neurons (V_{hold}, +10 mV). A photomicrograph of one of these neurons is shown in *3*. Scale bar, 50 μm. *2*, Pooled data (means ± SEM) illustrating a dose-dependent increase in the frequency of sIPSCs induced by 300 nM ($n = 6$), 1 μM ($n = 6$), and 10 μM ($n = 9$) ATPA ($^{**}p < 0.01$). LY293558 (30 μM), when applied alone, had no effect on the frequency of sIPSCs but prevented the ATPA-induced effect when coapplied with ATPA.

GluR5 kainate receptors are tonically activated by endogenous glutamate facilitating GABAergic synaptic transmission

To investigate whether presynaptic GluR5 kainate receptors of BLA interneurons are activated by endogenous glutamate, we examined the effects of bath application of LY293558 alone on the rate of failures of eIPSCs recorded from the soma of BLA pyramidal cells. LY293558 (30 μM) reversibly increased the percentage of failures of eIPSCs from 35.3 ± 11.2 to 67.0 ± 4.8% ($n = 8$; $p < 0.01$) (Fig. 8). This indicates that basal levels of extracellular glutamate escaping from excitatory synapses tonically modulate the activity of nearby inhibitory synapses via presynaptic GluR5 kainate receptors. This result is in contrast to the lack of an effect of LY293558, when applied alone, on the frequency of sIPSCs. One possible explanation for this apparent discrepancy is that the frequency of sIPSCs is regulated to a greater extent by the level of activation of somatodendritic GluR5 kainate receptors, rather than by GluR5 kainate receptors located on the terminals of the interneurons. Hence, the observation that

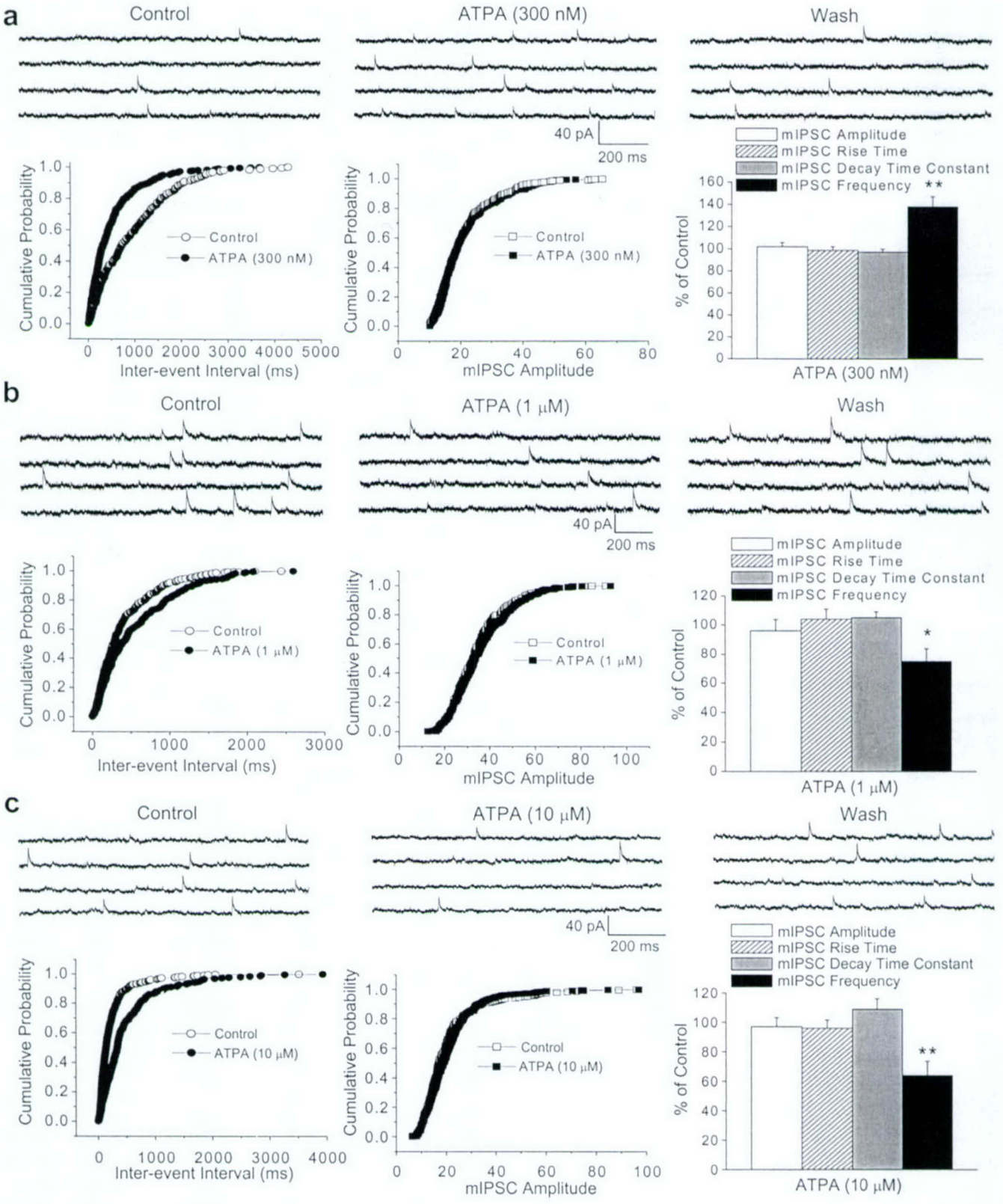

Figure 6. Dose-dependent, bidirectional modulation of the frequency of miniature GABAergic currents by the GluR5 agonist ATPA. *a–c, Top*, Samples of mIPSCs recorded from three different BLA pyramidal neurons before, during, and after the application of ATPA, at a 300 nM (*a*), 1 μM (*b*), or 10 μM (*c*) concentration. Recordings were obtained in the presence of TTX (1 μM), GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM), at a holding potential of +10 mV. *a–c, Bottom*, The corresponding cumulative probability plots of interevent intervals and amplitudes of mIPSCs in control conditions and during the application of ATPA. Bar graphs show pooled data (means ± SEM) on the effects of ATPA on mIPSCs. At 300 nM (*a*), ATPA increased the frequency of mIPSCs ($n = 9$; $^{**}p < 0.01$). At 1 μM (*b*), ATPA caused a small but significant reduction ($n = 7$; $^{*}p < 0.05$), and at 10 μM (*c*), ATPA caused a marked reduction in the frequency of mIPSCs ($n = 7$; $^{**}p < 0.01$). The peak amplitude, rise time, and decay time constant were not significantly affected by any of the three concentrations of ATPA. Perfusion of the slices with ATPA-free ACSF completely reversed the effects of the agonist.

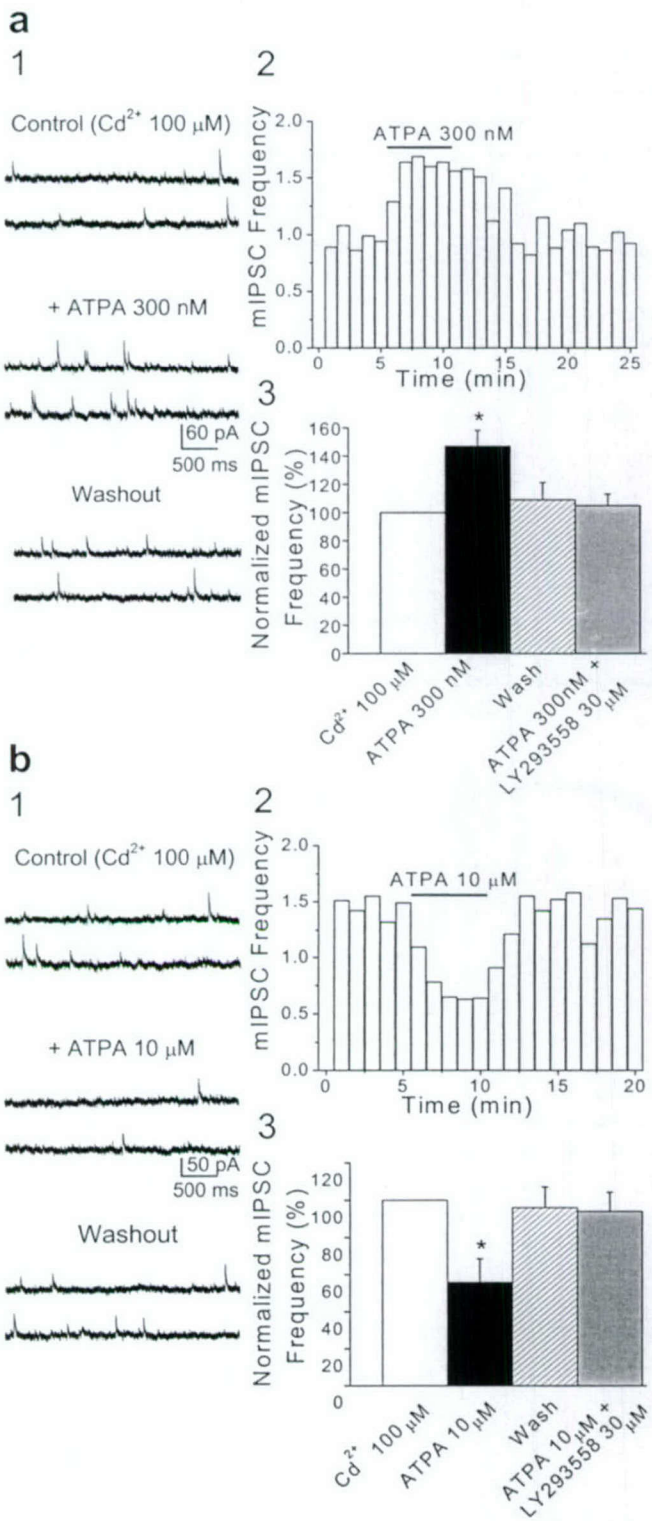

Figure 7. The effects of ATPA on the frequency of miniature GABAergic currents do not involve voltage-dependent calcium channels. *a1*, *b1*, Samples of mIPSCs recorded from two different BLA pyramidal neurons before, during, and after the application of 300 nM (*a1*) or 10 μM (*b1*) ATPA in the presence of Cd^{2+} (100 μM), TTX (1 μM), GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM), at a holding potential of +10 mV. The presence of Cd^{2+} in the medium did not prevent the increase (*a1*) or decrease (*b1*) in the frequency of mIPSCs by bath application of ATPA. *a2*, *b2*, Plots show the effects of 300 nM (*a2*) and 10 μM (*b2*) ATPA on the mean frequency of mIPSCs as a function of time (bin = 60 sec) (same cells as in *a1* and *b1*, respectively). *a3*, *b3*, Pooled data (means ± SEM) on the effects of ATPA, applied in the presence of Cd^{2+}, on the frequency of mIPSCs. *a3*, At 300 nM, ATPA increased the frequency of mIPSCs ($n = 6$; $^*p < 0.05$). *b3*, At 10 μM, ATPA caused a marked reduction in the frequency of mIPSCs ($n = 3$; $^*p < 0.05$). For each cell, the mIPSC frequency was normalized to the value of the mean mIPSC frequency before the application of ATPA. Coapplication of LY293558 (30 μM) prevented the effects of ATPA.

LY293558 increased the rate of failures of eIPSCs but did not affect the frequency of sIPSCs suggests either that extracellular glutamate reaches higher concentrations in the vicinity of presynaptic GluR5 kainate receptors than in the vicinity of somatodendritic GluR5 kainate receptors or that different subtypes of GluR5-containing kainate receptors are present in the somatodendritic versus the presynaptic sites, and the presynaptic GluR5 kainate receptors have a higher affinity for glutamate.

The GluR5 subunit is highly expressed in the basolateral amygdala

The agonist concentration-dependent, bidirectional modulation of GABA release via GluR5 receptors in the BLA is in contrast to findings in the hippocampus, where submicromolar concentrations of ATPA have no effect on evoked GABAergic transmission (Rodriguez-Moreno et al., 2000), and 1 or 10 μM ATPA induces only suppression (Clarke et al., 1997; Rodriguez-Moreno et al., 2000). In other regions, such as the dorsal horn, ATPA does not produce any significant effects on GABA release (Kerchner et al., 2001). Therefore, it appears that GluR5 kainate receptors may have different functions in different regions of the CNS; their function may depend on their location (cell type and subcellular compartment), subunit composition, and stoichiometry, as well as density. The density of the GluR5 subunit in the amygdala and particularly in the BLA, as revealed by *in situ* hybridization of GluR5 mRNA, is higher than in other CNS areas, including the hippocampus (Li et al., 2001) (Fig. 9) and the dorsal horn (Tölle et al., 1993). However, the mRNA levels are suggestive but not necessarily indicative of the expression levels of the GluR5 protein itself. For this reason, we performed a Western blot analysis of GluR5 protein expression levels using a specific antibody that recognizes GluR5 isoforms (Upstate Biotechnology). We found that the amygdala has higher expression levels of the GluR5 subunit compared with other brain regions, including the hippocampus (Fig. 9). The greater expression of the GluR5 subunit in the amygdala may imply a more prominent role of GluR5 kainate receptors in this structure.

Discussion

Presynaptic GluR5 kainate receptors mediate both facilitation and suppression of GABAergic transmission

The main finding of this study is that in interneuron-to-pyramidal-cell synapses in the basolateral amygdala, low concentrations of GluR5 kainate receptor agonists facilitate, whereas high concentrations suppress, GABAergic transmission. In addition, ambient concentrations of extracellular glutamate tonically facilitate inhibitory transmission. Both facilitation and suppression of inhibitory transmission are induced by a direct effect of GluR5 agonists on GABAergic terminals, as indicated by the effects of ATPA on mIPSCs, and do not require the activation of voltage-gated calcium channels or presynaptic $GABA_B$ receptors.

The most plausible interpretation of the dose-dependent, bidirectional effects of GluR5 agonists on GABAergic transmission is that the terminals of GABAergic neurons in the BLA carry two subtypes of GluR5-containing kainate receptors, which have different affinities for their agonists and activate different mechanisms of action. Based on their affinity for [^{3}H]kainate, kainate receptor subunits can be divided into low-affinity (GluR5, GluR6, and GluR7) and high-affinity (KA1 and KA2) subunits (Chittajallu

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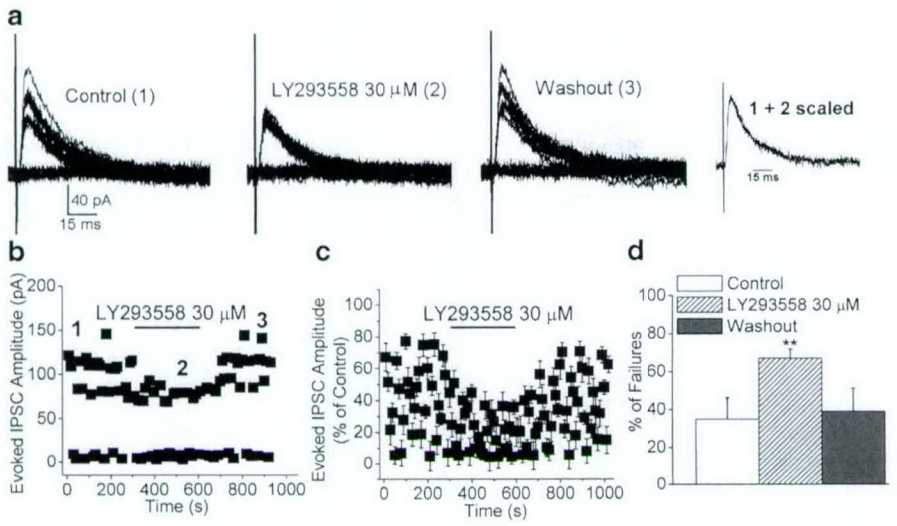

Figure 8. Endogenous glutamate tonically activates GluR5 kainate receptors, facilitating GABAergic synaptic transmission. *a*, Superimposed traces of eIPSCs recorded from a BLA pyramidal neuron before, during, and after the application of LY293558 (30 μM). Recordings were obtained in the presence of GYKI 53655 (50 μM), D-APV (50 μM), and SCH50911 (20 μM), at a holding potential of +10 mV. The scaled superimposed traces show that the effects of LY293558 (30 μM) were not accompanied by changes in the kinetics of the eIPSCs. *b*, The time course of the effects of LY293558 (30 μM) on the amplitude and number of failures of eIPSCs (same cell as in *a*). *c*, Pooled data (means ± SEM) illustrating a marked increase in the percentage of failures of eIPSCs induced by bath application of LY293558 ($n = 8$; $^{**}p < 0.01$).

et al., 1999). Our previous work has shown that the basolateral nucleus of the amygdala also expresses the GluR6 and KA2 subunit mRNAs in addition to GluR5 mRNA (Li et al., 2001). There is also evidence that the GluR5 subunit can form functional kainate receptors with GluR6 or KA2 subunits, and both GluR5/GluR6 and GluR5/KA2 kainate receptors are sensitive to ATPA (Paternain et al., 2000). Therefore, a GluR5/KA2 and a GluR5/GluR6 subunit combination could mediate the facilitation and inhibition, respectively, of GABAergic transmission in the BLA. Consistent with the view that a GluR5/GluR6 subunit combination may mediate the suppression of GABAergic transmission in the BLA, Mulle et al. (2000) found that kainate-induced suppression of eIPSCs in the hippocampus is mediated by heteromeric kainate receptors composed of both GluR5 and GluR6 subunits.

The intracellular mechanisms activated by these receptors remain to be determined. GluR5-containing kainate receptors can be permeable to Ca^{2+}, particularly when they contain the unedited version of the GluR5 subunit, which confers higher Ca^{2+} permeability (Burnashev et al., 1996; Savidge et al., 1997; Chittajallu et al., 1999). In the amygdala, ~30% of the total GluR5 mRNA is in the unedited form (Li et al., 2001). Therefore, a substantial portion of the GluR5-containing kainate receptors in the amygdala must have a relatively high permeability to Ca^{2+}. Thus, some of the observed effects, more likely the facilitation of GABAergic transmission, could be mediated by Ca^{2+} influx through GluR5 kainate receptors on GABAergic terminals. The mechanisms responsible for the suppression of GABAergic transmission by GluR5 kainate receptor activation in the amygdala may involve a metabotropic cascade, as suggested previously for the kainate receptor-mediated inhibition of transmitter release in hippocampal synaptosomes (Cunha et al., 2000), as well as in interneuron-to-pyramidal-cell synapses (Rodriguez-Moreno and Lerma, 1998) or in glutamatergic synapses (Frerking et al., 2001) in the CA1 hippocampal area.

Physiological relevance of the bidirectional modulation of GABA release by glutamate

The results presented here suggest a significant role of glutamate diffusion in the regulation of excitability in the amygdala by modulating GABA release via presynaptic, GluR5-containing kainate receptors. Low concentrations of extracellular glutamate escaping from excitatory synapses during low or moderate activity of excitatory pathways within the amygdala neuronal network can be expected to facilitate GABAergic transmission. Our data demonstrate that even ambient concentrations of extracellular glutamate released during unstimulated, basal activity of excitatory synapses can reach and activate GluR5 kainate receptors present on GABAergic terminals, increasing the efficacy of GABAergic synapses. Considering the central role of the amygdala in fear conditioning and the consolidation of emotional memories (Aggleton, 2000; McGaugh, 2002), such facilitation of GABAergic transmission may serve to prevent or dampen the excitation of the amygdala dur-

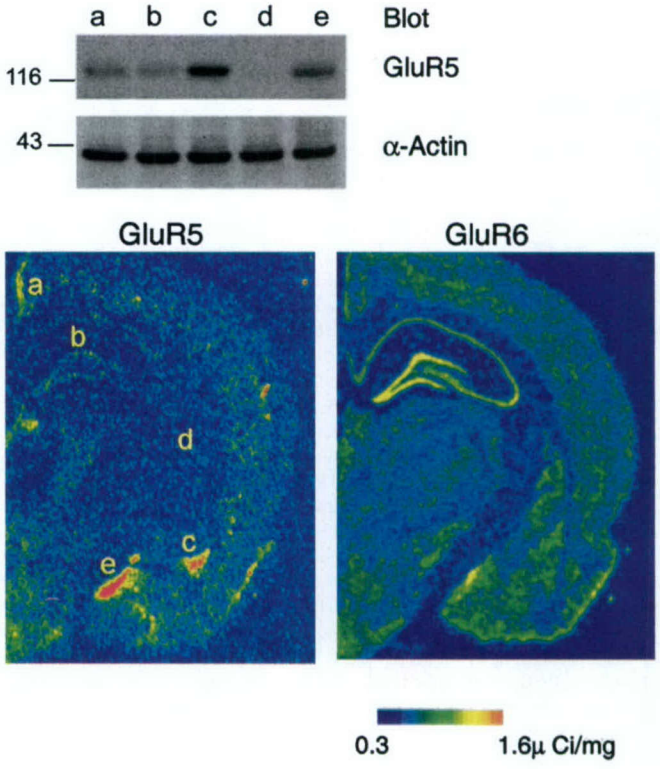

Figure 9. The basolateral amygdala has a high expression of the GluR5 protein. Western blot analysis of the GluR5 protein levels (*top*) in five brain regions: *a*, retrosplenial granular cortex; *b*, hippocampus; *c*, basolateral amygdala; *d*, medial globus pallidus; *e*, medial amygdala. These regions are indicated in the *pseudocolor image* of GluR5 mRNA *in situ* hybridization data from rat brain (from Li et al., 2001). An *in situ* hybridization *pseudocolor image* of GluR6 mRNA (from Li et al., 2001) is also shown for comparison. Although GluR6 mRNA signal is strongest in the hippocampus, GluR5 mRNA and GluR5 protein levels are highest in the amygdala. Parallel immunoblotting for α-actin was used to verify the equal loading of cell lysates. The molecular mass is indicated.

ing external or internal stimuli that have only moderate emotional significance. In contrast, in response to the intense emotional stimuli that produce strong excitation of the amygdala, the amount of glutamate released may reach sufficiently high extrasynaptic concentrations to activate the low-affinity GluR5 kainate receptors on GABAergic terminals, inhibiting evoked GABAergic transmission. Because spontaneous GABA release may be enhanced (Fig. 5*b*) at the same time that evoked GABA release is suppressed, depending on the accessibility of somatodendritic versus presynaptic GluR5 receptors to extrasynaptic glutamate, the question arises as to what would be the net effect of high concentrations of extrasynaptic glutamate on GluR5-mediated changes in GABAergic activity and, therefore, on the overall excitability of the neuronal circuits of the amygdala. High concentrations of ATPA (>5 μM) produce a dramatic increase in the excitability of the amygdala, as revealed in both intracellular (Li et al., 2001) and field potential recordings (V. Aroniadou-Anderjaska, unpublished observations). This suggests that the reduction of evoked GABAergic transmission when GluR5 receptors are exposed to high concentrations of extracellular glutamate overrides any increases in spontaneous GABA release. Such an effect could further enhance overactivity in the amygdala during intense emotional stimuli, and perhaps facilitate the "registration" of the memory trace representing the emotional event. In this respect, the GluR5-mediated disinhibitory effect of glutamate may play an important role in synaptic plasticity and memory formation in the amygdala, as well as in the development of certain stress-induced affective disorders such as post-traumatic stress syndrome. Finally, the GluR5 kainate receptor-mediated disinhibitory action of glutamate described here sheds light on the mechanisms of the epileptogenic and neurotoxic actions of kainate in the amygdala.

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SEROTONIN TYPE II RECEPTOR ACTIVATION FACILITATES SYNAPTIC PLASTICITY VIA *N*-METHYL-D-ASPARTATE-MEDIATED MECHANISM IN THE RAT BASOLATERAL AMYGDALA

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Abstract—The modulation of synaptic plasticity by serotonin type II (5-hydroxytryptamine type II (5-HT_2))-receptor stimulation was explored using intracellular, field potential and Fura-2 fluorescence image recordings in a rat amygdala slice preparation. Bath application of $5HT_2$ receptor agonist 1-(2,5)-dimethoxy-4-iodophen-2-aminopropane (DOI) transformed θ-burst-stimulated (TBS) synaptic plasticity from short-term potentiation to long-term potentiation. DOI enhanced *N*-methyl-D-aspartate (NMDA) receptor-mediated potentials and calcium influx without affecting the resting membrane potential or input resistance of the neurons. In contrast, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptor-mediated excitatory synaptic responses were unaffected by DOI. The facilitating effects of DOI were blocked by the 5-HT_2 receptor antagonist, ketanserin, and by the 5-HT_{2C}-receptor selective antagonist, RS102221. These results indicate that 5-HT_2-receptor activation enhances NMDA receptor-mediated synaptic function in the basolateral amygdala (BLA). Published by Elsevier Science Ltd on behalf of IBRO.

Key words: DOI, serotonin, synaptic plasticity, LTP, calcium, fear.

The amygdala complex is well known for its involvement in mood and emotion (Davis, 1992; Ledoux, 1995), and may participate in the pathogenesis of schizophrenia (Bogerts et al., 1985), depression (Drevets et al., 1992), epilepsy (Boucsein et al., 2001; Pitkanen et al., 1998) and posttraumatic stress disorder (PTSD) (Liberzon et al., 1999; Post et al., 1998; Rauch et al., 2000). In these illnesses, the importance of serotonergic neurotransmission is universally acknowledged. Enhanced serotonin release during periods of anxiety and stress has been observed in the amygdala complex (Fernandes et al., 1994). The amygdala expresses high levels of serotonin type II (5-hydroxytryptamine type II (5-HT_2)) receptor protein and mRNAs (Morilak et al., 1994; Pompeiano et al., 1994; Wright et al., 1995). Activation of 5-HT_2 receptors in the amygdala facilitates the development of amygdaloid kindling (Wada et al., 1997) and has a profound effect upon anxiety and mood (Hrdina et al., 1993; Kshama et al., 1990; Tokuyama et al., 1993).

Excitatory neurotransmission in the basolateral amygdala (BLA) is mediated by *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptors (Li and Rogawski, 1998; Rainnie et al., 1991). This excitatory neurotransmission exhibits NMDA receptor-dependent and -independent long-term synaptic plasticity (Gean et al., 1993; Li et al., 1998, 2001; Maren, 1999). Synaptic plasticity can be negatively modulated in the visual cortex by 5-HT_2-receptor stimulation (Edagawa et al., 2000). The effect of 5-HT_2-receptor modulation of synaptic plasticity of amygdala circuitry, however, remains to be elucidated. This synaptic plasticity may underlie the learning of traumatic memories that characterize fear conditioning, anxiety disorders and posttraumatic stress disorder.

In the study reported here, we characterize the excitatory effect of 5-HT_2-receptor stimulation in the basolateral amygdala, using electrophysiological and calcium imaging techniques. The results demonstrate that 5-HT_2-receptor activation facilitates NMDA receptor-dependent synaptic potentiation in the basolateral amygdala (BLA) by enhancing NMDA receptor-mediated calcium influx.

EXPERIMENTAL PROCEDURES

Amygdala slice preparation

Male Sprague–Dawley rats weighing 75–150 g (4–6 weeks) were used. The rats were decapitated, the brains rapidly removed, and transverse slices (500 μm thickness for intracellular recording and field potential recording, 350 μm thickness for calcium imaging) of the amygdala were cut from tissue blocks with a Vibratome (Technical Products International, St. Louis, MO, USA). The slices were preincubated in oxygenated artificial cerebrospinal fluid (ACSF) continuously bubbled at room temperature (25 °C) with 95% O_2/5% CO_2 for at least 1 h before use. The ACSF contained (in mM) 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose, and was bubbled with 95% O_2/5% CO_2 to maintain a pH of 7.4. Experiments carried out in amygdala

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Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate; APV, (±)-2-amino-5-phosphonovaleric acid; Bic, bicuculline methochloride; BLA, basolateral amygdala; DOB, (−)-1-2,5-dimethoxy-4-bromophenol-2-aminopropane; DOI, (±)-1-(2,5-dimethoxy-4-iodophen)-2-aminopropane hydrochloride; EC, external capsule; EPSP, excitatory postsynaptic potentials; Fura-2 AM, fura-2 acetoxymethyl ester; 5-HT_2, 5-hydroxytryptamine type II; LTP, long-term potentiation; LY293558, {(3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazol-5-yl)ethyl]-decahydroisoquinoline-3-carboxylic acid}; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; PPF, paired-pulse facilitation; PTSD, posttraumatic stress disorder; RS102221, 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulfonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride; SCH50911, (2s)-(+)-5,5-dimethyl-2-morpholineacetic acid; TBS, θ-burst stimulation; WCP, Whole Cell Electrophysiology Program.

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GluR5 Kainate Receptors, Seizures, and the Amygdala

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ABSTRACT: **The amygdala is a critical brain region for limbic seizure activity, but the mechanisms underlying its epileptic susceptibility are obscure. Several lines of evidence implicate GluR5 (GLU_{K5}) kainate receptors, a type of ionotropic glutamate receptor, in the amygdala's vulnerability to seizures and epileptogenesis. GluR5 mRNA is abundant in temporal lobe structures including the amygdala. Brain slice recordings indicate that GluR5 kainate receptors mediate a portion of the synaptic excitation of neurons in the rat basolateral amygdala. Whole-cell voltage-clamp studies demonstrate that GluR5 kainate receptor-mediated synaptic currents are inwardly rectifying and are likely to be calcium permeable. Prolonged activation of basolateral amygdala GluR5 kainate receptors results in enduring synaptic facilitation through a calcium-dependent process. The selective GluR5 kainate receptor agonist ATPA induces spontaneous epileptiform bursting that is sensitive to the GluR5 kainate receptor antagonist LY293558. Intra-amygdala infusion of ATPA in the rat induces limbic status epilepticus; in some animals, recurrent spontaneous seizures occur for months after the ATPA treatment. Together, these observations indicate that GluR5 kainate receptors have a unique role in triggering epileptiform activity in the amygdala and could participate in long-term plasticity mechanisms that underlie some forms of epileptogenesis. Accordingly, GluR5 kainate receptors represent a potential target for antiepileptic and antiepileptogenic drug treatments. Most antiepileptic drugs do not act through effects on glutamate receptors. However, topiramate at low concentrations causes slow inhibition of GluR5 kainate receptor-mediated synaptic currents in the basolateral amygdala, indicating that it may protect against seizures, at least in part, through suppression of GluR5 kainate receptor responses.**

KEYWORDS: **amygdala; GluR5 kainate receptor; AMPA receptor; epilepsy; ATPA; LY293558; topiramate; synaptic transmission; synaptic plasticity**

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INTRODUCTION

The amygdala is of special interest in relation to mechanisms of epilepsy because it is part of the medial temporal structures that are often involved in human complex partial seizures.[1] In addition, the amygdala is a key site for kindling in animals.[2,3] Through its connections with the entorhinal cortex and hippocampus, the amygdala plays a pivotal role in the generation and spread of limbic seizure activity. However, the cellular mechanisms that underlie the epileptic susceptibility of the amygdala are only beginning to be understood. The circuitry of the amygdala seems to favor synchronized firing of the type that is required for epileptic discharges.[4] Additionally, activity-dependent synaptic plasticity mechanisms (possibly triggered during kindling) likely play a role in modifying the efficacy of certain amygdala synapses, leading to a bias towards paroxysmal epileptic activity.[5] A diversity of neurotransmitter mechanisms undoubtedly participate in amygdala epileptogenenesis. On the basis of studies in the *in vitro* amygdala slice, we have developed evidence that GluR5 kainate receptors, a type of ionotropic glutamate receptor, plays a unique role in triggering seizure discharges. Our studies have focused on the basolateral amygdala (BLA), which is an amygdala nucleus that is highly susceptible to epileptogenesis.[6,7] Here we review recent experiments addressing the anatomical localization and functional roles of GluR5 kainate receptors in the BLA. These studies demonstrate that GluR5 kainate receptors participate in synaptic transmission and synaptic plasticity in the BLA. In addition, we found that selective activation of amygdala GluR5 kainate receptors elicits synchronized bursting of BLA neurons *in vitro* and limbic seizure activity in the intact animal. Overall, our observations implicate GluR5 kainate receptors as a substrate for amygdala epileptic activity.

KAINATE AND AMYGDALA EPILEPTOGENESIS

Kainate, a neuroexcitant and excitotoxin derived from the marine algae *Digenea simplex*, is an agonist of some ionotropic glutamate receptors and is well recognized as a proconvulsant substance.[8] Although other glutamate receptor agonists can induce intense convulsive seizures when administered to animals, kainate is unique among the common agonists (e.g., NMDA, AMPA) in that low systemic doses produce prolonged limbic seizures (wet-dog shakes, facial and forelimb clonus, and rearing).[9] A high proportion of animals surviving such an attack of limbic status epilepticus go on to have spontaneous seizures throughout their lives.[10] Thus, in addition to its proconvulsant ("seizure-inducing") properties, kainate is epileptogenic in that it can induce a long-lasting transformation to a seizure-susceptible state. The seizures occurring in kainate-kindled animals are of the limbic type, suggesting involvement of temporal lobe structures including the amygdala. Moreover, it is well recognized that amygdala neurons are particularly sensitive to excitotoxic damage by kainate.[11,12] Therefore, the amygdala is a likely target site for the pro-epileptic action of kainate. In fact, prolonged limbic status epilepticus is produced by focal intra-amygdaloid infusion of kainate.[13] After a variable latent period of 2 weeks or more in which the animals exhibit interictal discharges but no behavioral seizures, spontaneous limbic and secondarily generalized seizures may occur.[14] Therefore, kainate-induced activation of the amygdala region alone is sufficient for limbic epileptogenesis.

KAINATE RECEPTORS

Fast excitatory neurotransmission in the mammalian central nervous system is mainly mediated by glutamate acting on NMDA, AMPA, and kainate ionotropic glutamate receptors. These receptors are multi-subunit (probably tetrameric) membrane proteins that act as cation channels.[15] In response to synaptically released glutamate, they permit sodium (and in some cases also calcium) to enter into the neuron, generating depolarization and excitation. Recently, we demonstrated through the use of selective pharmacological antagonists[16] that kainate receptors play a role in excitatory neurotransmission in the amygdala.[17] The subunit proteins that constitute kainate receptors are termed GluR5, GluR6, GluR7, KA1, and KA2 (more recent IUPHAR nomenclature GLU_{K5}–GLU_{K7}, GLU_{K1}, and GLU_{K2}). These receptor subunits have molecular masses of ~100 kDa (approximately 900 amino acids) and, based upon their amino acid sequences, are ~40% homologous to AMPA receptors and ~20% homologous to NMDA receptors.

Although there is only limited information on the molecular diversity of kainate receptors in brain neurons, there are a number of ways such diversity could be generated. GluR5-7 and KA1-2 subunits can combine in various stoichiometries to form functionally distinct heteromeric kainate receptor subtypes. (KA1 and KA2 do not form functional channels by themselves.) In addition, individual subunits can exist in alternatively spliced forms. GluR5 and GluR6 kainate receptors are susceptible to structural modification through mRNA editing, as occurs for the GluR2 AMPA receptor subunit. The subunit pre-mRNA is believed to undergo posttranscriptional revision at specific sites so that it encodes a different amino acid at these sites from the one coded by the gene. A particularly critical site for such editing is the glutamine/arginine (Q/R) site in the M2 segment, which regulates the permeability properties of the receptor. In the case of homomeric GluR6 receptors, the Q-to-R substitution decreases the permeability to calcium and transforms the rectification properties from inwardly rectifying to linear or slightly outwardly rectifying. Similarly, heteromeric receptors containing an edited GluR5 or GluR6 subunit are calcium-impermeable and linear or outwardly rectifying. Therefore, calcium-permeable kainate receptors exclusively contain unedited GluR5 or GluR6 subunits.

MODERN PHARMACOLOGY OF KAINATE RECEPTORS

Many kainate receptor agonists, including the natural agonist glutamate, nonselectively activate AMPA receptors as well as kainate receptors. Kainate, the prototypic agonist for kainate receptors, induces kainate receptor currents that desensitize over the course of several hundred milliseconds, even in the continued presence of the agonist.[18] However, kainate also activates AMPA receptors at similar concentrations (EC_{50} ~150 μM), eliciting currents that do not desensitize.[19] Thus, kainate cannot be used to selectively activate kainate receptors. Domoic acid, found naturally in marine phytoplankton diatoms such as *Pseudo-nitzschia multiseries* (a cause of shellfish poisoning), is approximately 10-fold more potent than kainic acid as an agonist of kainate receptors.[20] However, like kainate, it activates AMPA receptors.[19]

Newer pharmacological tools permit the roles of kainate receptors to be characterized without confounding effects of AMPA receptor activation. For example, several AMPA/kainate agonists have been identified that preferentially activate kainate receptors containing the GluR5 subunit. One such agonist is ATPA, a *tert*-butyl analog of AMPA.[21] ATPA is a potent agonist of recombinant homomeric and heteromeric GluR5 kainate receptors (EC_{50} values, 0.6–2 μM),[22,23] but a weak, partial agonist at AMPA receptors and GluR6/KA2 kainate receptors.[24,25] Therefore agonists such as ATPA permit GluR5 kainate receptors to be selectively activated. 2,3-Benzodiazepine non-NMDA antagonists including GYKI 53655 and GYKI 52466 are an additional set of tools that have been critical in the study of kainate receptors.[26] These noncompetitive (allosteric) antagonists preferentially block AMPA receptors and not kainate receptors, with a selectivity of >10- to 200-fold.[18,27] Thus, at appropriate concentrations, these drugs largely eliminate AMPA receptor-mediated responses, allowing kainate responses to be studied in isolation. Decahydroisoquinolines, such as LY293558, LY296486, and LY382884, make it possible to confirm that a response obtained in the presence of AMPA receptor blockade is mediated specifically by GluR5 kainate receptors.[21,28–30] These compounds have varying degrees of AMPA receptor blocking activity and also were recently demonstrated to inhibit homomeric or heteromeric kainate receptors containing at least one GluR5 subunit. They have no detectable activity at homomeric GluR6 kainate receptors.

GLUR5 KAINATE RECEPTORS MEDIATE EXCITATORY SYNAPTIC TRANSMISSION IN THE AMYGDALA

In situ hybridization studies have demonstrated that the various kainate receptor subunit mRNAs are widely expressed within the nervous system in a nonuniform distribution.[31] The mRNAs have been found in neocortex, limbic system, and cerebellum. Recently, we noted that GluR5 mRNA is strongly expressed in temporal lobe structures, including the amygdala and piriform cortex.[32] These kainate receptors have a variety of roles in synaptic transmission. At some synapses, kainate receptors mediate a portion of the glutamatergic postsynaptic response, whereas at other synapses, kainate receptors act as presynaptic modulators of synaptic release. (For reviews, see Refs. 33 and 34.) In the BLA, the bulk of the glutamatergic excitatory postsynaptic response is mediated by AMPA receptors. However, using selective pharmacological antagonists, we found that GluR5 kainate receptors are responsible for a component (about 30%) of the fast synaptic depolarization ("excitatory postsynaptic potential" or EPSP).[17,32] The GluR5 kainate receptor component, isolated in the presence of the selective AMPA receptor antagonist GYKI 52466 and recorded under voltage-clamp conditions, is shown in FIGURE 1A,B. (The recording solution also contained antagonists of NMDA, $GABA_A$, and $GABA_B$ receptors.) Note that very low concentrations of LY293558 can completely eliminate the kainate receptor-mediated synaptic current; these low concentrations do not substantially affect pure AMPA receptor-mediated synaptic currents. Interestingly, the GluR5 kainate receptor synaptic current has inwardly rectifying properties that are apparent from the current-voltage relationship (not shown). This suggests that at least some of the multimeric GluR5 kainate receptors exclusively contain GluR5 subunits that

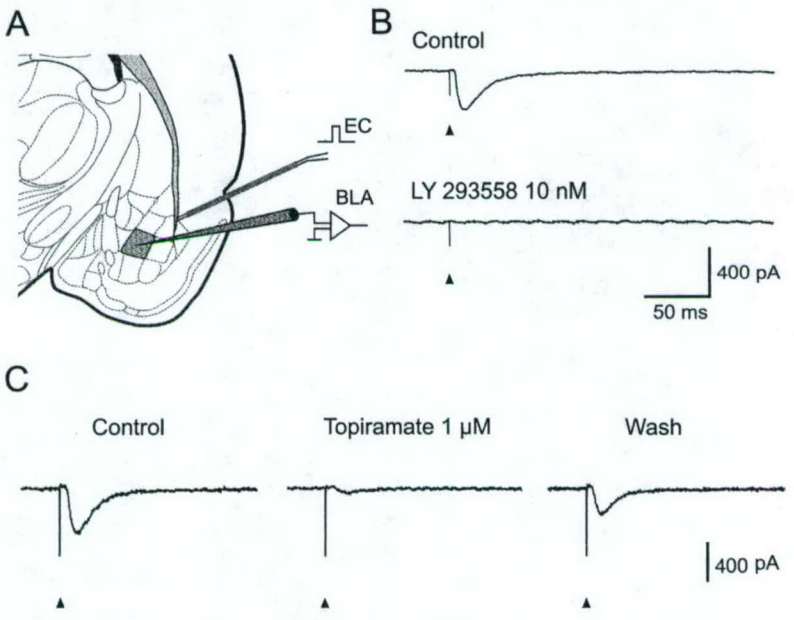

FIGURE 1. GluR5 kainate receptor-mediated excitatory synaptic transmission in the basolateral amygdala (BLA): block by the anticonvulsant topiramate. **(A)** Schematic illustration of the configuration for whole-cell voltage-clamp recording in the rat amygdala slice (coronal section). A tungsten bipolar stimulating electrode placed on the external capsule (EC) is used to deliver 100 µs-duration monophasic stimuli. Patch recordings are obtained from visually identified principal neurons in the BLA. GluR5 kainate receptor responses are isolated by perfusion with 100 µM D-AP5, 50 µM GKYI 52466, 10 µM bicuculline methiodide, and 10 µM SCH 50911 to block NMDA, AMPA, $GABA_A$, and $GABA_B$ receptors, respectively. **(B)** Control trace is the GluR5 kainate receptor-mediated component of EC-evoked synaptic current. The response is rapidly eliminated by addition of 10 nM LY293558, which at these low concentrations selectively blocks GluR5 kainate receptors. Holding potential, –120 mV. **(C)** Topiramate causes a slow block of GluR5 kainate receptor-mediated synaptic current. Fifty-two minutes after onset of perfusion with 1 µM topiramate, the current is largely eliminated (*middle trace*). *Right trace* shows partial recovery 12 minutes after removal of topiramate from the perfusion solution. Holding potential, –70 mV.

are unedited at the Q/R site. In fact, we found that a proportion of GluR5 kainate receptor mRNAs in the BLA are unedited.[32] Such inwardly rectifying GluR5 kainate receptors are expected to be calcium permeable. This property of BLA GluR5 kainate receptors could indicate a role in forms of synaptic plasticity, as is the case for some other calcium-permeable ionotropic receptors such as NMDA and calcium-permeable AMPA receptors.[35]

ACTIVATION OF GLUR5 KAINATE RECEPTORS INDUCES EPILEPTIFORM DISCHARGES

It is well recognized that low concentrations of kainate (1 μM) can induce spontaneous epileptiform activity in *in vitro* brain slice preparations of hippocampus and neocortex.[36–38] However, whether the proconvulsant activity of kainate occurs through activation of kainate or AMPA receptors is uncertain. We used ATPA in an *in vitro* amygdala slice preparation to address the relative roles of kainate and AMPA receptor activation in the induction of epileptiform activity in the amygdala. We found that bath application of ATPA elicited spontaneous synchronized bursting in the BLA within 5–10 minutes of the onset of drug perfusion. The threshold concentration was 2.5 μM, and robust responses were observed at 10 μM (FIG. 2). By contrast, AMPA at comparable concentrations usually led to termination of all activity, presumably because of depolarization inactivation. Similarly, in the BLA, kainate also generally led to termination of activity, which can be attributed to its AMPA receptor agonist activity. Coadministration of the decahydroisoquinoline GluR5 kainate receptor antagonist LY293558 at concentrations of 100–250 nM reduced the frequency of ATPA-induced bursting and terminated bursting at 500 nM. Taken together, these results demonstrate that activation of GluR5 kainate receptors can induce epileptiform discharges. The results also suggest that the *in vitro* pro-epileptic activity of kainate may relate to effects specifically on kainate receptors and not AMPA receptors.

GLUR5 KAINATE RECEPTORS AS TARGETS FOR ANTIEPILEPTIC DRUGS

Ionotropic glutamate receptor antagonists are effective in protecting against various types of seizures in animal models. As a consequence, there has been considerable interest in their potential use in epilepsy therapy.[39,40] However, attempts to develop NMDA receptor antagonists for epilepsy therapy have not been encouraging, and to date, only preliminary clinical trials have been carried out with AMPA receptor antagonists. Inasmuch as selective activation of kainate receptors elicits epileptiform activity (at least in the BLA), it is conceivable that blockade of kainate receptors could suppress seizure activity. In this case, kainate receptors may represent a promising anticonvulsant drug target.

No currently marketed anticonvulsant is believed to interact directly with ionotropic glutamate receptors, with the possible exception of topiramate, which has been reported to block kainate-activated currents in cultured hippocampal neurons.[41] In these prior studies, the specific receptor type targeted by kainate was not established. We found that topiramate, at low concentrations, selectively inhibits GluR5 kainate receptor-mediated synaptic currents in the BLA, but is much less effective against AMPA receptor responses (FIG. 1C). Topiramate block of GluR5 kainate receptor responses was slow, suggesting that it could act indirectly, perhaps affecting second-messenger mechanisms that modulate the activity of kainate receptors. In fact, studies with recombinant kainate receptors in a heterologous expression system have confirmed that the drug does not directly affect the GluR5 kainate receptor channel complex.[42] Evidence indicates that topiramate may modify the phos-

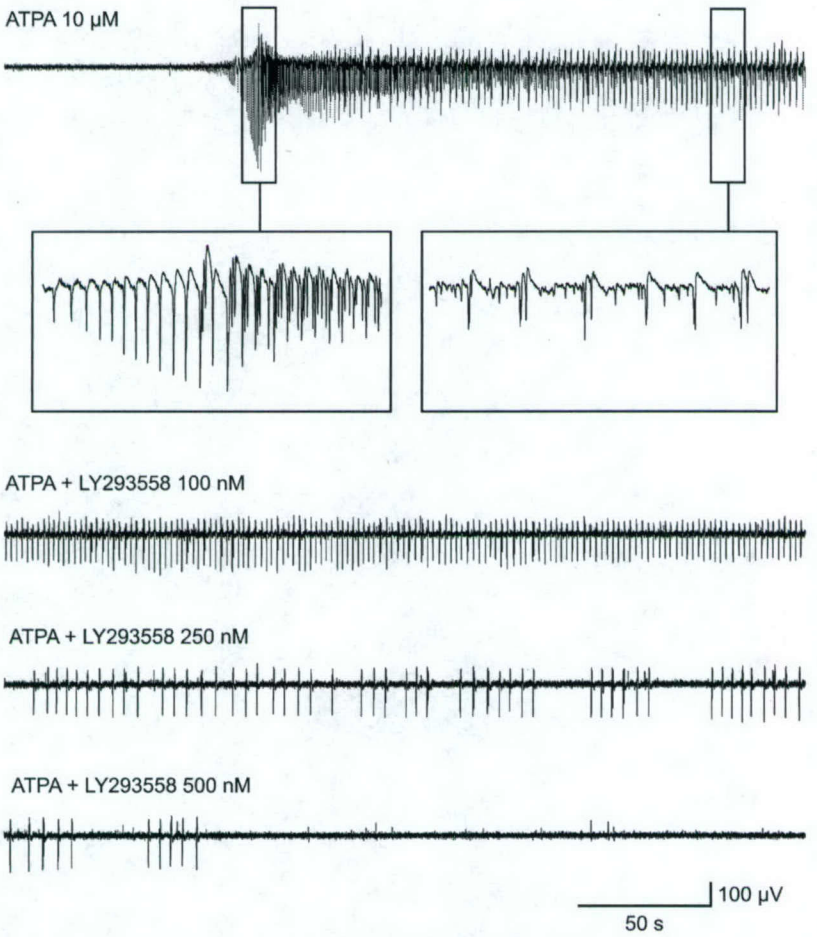

FIGURE 2. Epileptiform activity induced by the GluR5 kainate receptor agonist ATPA in the rat amygdala slice and its block by the GluR5 antagonist LY293558. Extracellular recordings were made from the basolateral amygdala (BLA) in a rat amygdala slice at 31°C. Bath perfusion with 10 µM ATPA elicits spontaneous epileptiform bursts within 10 minutes of the onset of drug application (*top*). Co-perfusion with increasing concentrations of LY293558 results in a reduction in the frequency of bursting and, at 500 nM, cessation of activity. *Boxed areas* in the *top trace* are shown on an expanded time scale in the two insets.

phorylation state of proteins.[43] It will be of interest to determine if this action contributes to its effects on kainate receptors. In any case, the demonstration that topiramate, a highly effective, broad spectrum, antiepileptic agent, can block kainate receptors at low concentrations supports the concept that kainate receptors may represent a promising target for antiepileptic drug development.

ROLE OF KAINATE RECEPTORS IN SYNAPTIC PLASTICITY

The strength of synaptic transmission at amygdala synapses can be modified in an activity-dependent fashion, a phenomenon generally referred to as "synaptic plasticity." Various forms of synaptic plasticity have been described in the amygdala. Some are associated with brief changes in synaptic efficacy, whereas others are enduring. Long-term potentiation (LTP), the prototypical form of enduring synaptic facilitation in the mammalian brain, which has been extensively studied in the hippocampus and neocortex, also occurs in the amygdala,[44–46] where it is believed to play a role in fear conditioning.[47] LTP in the amygdala is typically elicited by a brief high-frequency tetanus. As in other brain regions, LTP in the amygdala is synapse specific and is often dependent on calcium entry through NMDA receptors,[48] but it has also been linked to entry of calcium through voltage-gated calcium channels.[49]

Recently, we reported that low frequency stimulation (LFS) of excitatory afferents to BLA neurons can induce a novel form of enduring synaptic facilitation[50] that, like conventional LTP, requires calcium entry.[32] However, LFS-induced enduring synaptic facilitation has many characteristics that are distinct from LTP. Importantly, this novel form of synaptic facilitation is not dependent on NMDA receptors, but instead requires activation of GluR5 kainate receptors. Thus, decahydroisoquinoline GluR5 kainate receptor antagonists, but not NMDA receptor antagonists, prevent the induction of LFS-induced synaptic facilitation in the BLA. In addition, direct activation of GluR5 kainate receptors with ATPA can cause a robust and long-lasting enhancement in the synaptic response reminiscent of the effects of stimulation. LFS-induced synaptic facilitation develops slowly (over the course of ~15 minutes) in contrast to conventional LTP in which the potentiated response is seen immediately after termination of the stimulation. This suggests that LFS-induced synaptic facilitation may have a distinct mechanistic basis. It is now well recognized that conventional LTP is triggered by postsynaptic receptor activation and may be expressed postsynaptically through an increase in the number of AMPA receptors that contribute to the response. By contrast, whereas LFS-induced synaptic facilitation also appears to be induced postsynaptically, it could be expressed presynaptically through enhanced glutamate release. Another critical difference is that LFS-induced synaptic facilitation is not synapse specific. Inputs onto the target cell other than the pathway stimulated may be facilitated. Such nonspecific enhancement of excitatory inputs could conceivably be a mechanism whereby activation of GluR5 kainate receptors leads to the progressive spread of enhanced excitability, resulting in the conversion to an epileptic state.

ROLE OF KAINATE RECEPTORS IN EPILEPTOGENESIS

Using the selective AMPA receptor antagonist GKYI 52246 and the mixed AMPA/GluR5 antagonist LY293558, we examined whether conventional amygdaloid kindling in the mouse (daily stimulation with a single 1-s duration 60-Hz train) requires activation of GluR5 kainate receptors. Our experiments demonstrate that GluR5 kainate receptor activation is not obligatory for this type of kindling.[51] AMPA/kainate receptor blockers do protect against the expression of amygdala-kindled seizures; however, such antagonists do not appear to slow the rate at which kin-

dling is induced. Rather, induction of this form of kindling seems to be uniquely sensitive to NMDA receptor blockade. On the other hand, it is conceivable that some forms of amygdala epileptogenesis do result from kainate receptor activation. In preliminary experiments we observed that a single intra-amygdala infusion of the GluR5 kainate receptor agonist ATPA in the rat induces limbic status epilepticus and, in some animals, recurrent spontaneous seizures that occur paroxysmally for months after the infusion.[52]

PERSPECTIVE

The various lines of evidence presented here indicate that GluR5 kainate receptor activation can trigger seizures in the amygdala. Because the amygdala is a key substrate for temporal lobe seizures in humans,[1] drugs that target GluR5 kainate receptors could potentially be of value in the treatment of temporal lobe epilepsy. The distribution of GluR5 is more restricted than is that of GluR6. Therefore, selective GluR5 antagonists would be expected to have less neurological and behavioral toxicity than would nonselective kainate receptor antagonists.

Outside the amygdala, GluR5 is likely to be less important in seizure regulation than GluR6, which has more widespread expression in structures relevant to seizures, including the neocortex and hippocampus.[32] Therefore, mice in which the GluR6 gene is disrupted by homologous recombination have reduced (but not absent) sensitivity to the convulsant effects of systemic (intraperitoneal) kainate.[53] Conversely, mice engineered to be deficient in GluR6 Q/R-site editing and therefore express only calcium-permeable GluR6 receptors have enhanced kainate convulsant sensitivity.[54] GluR5 kainate receptors are unlikely to play a significant role in the convulsant activity of systemic kainate, because mice engineered to express only edited (calcium-impermeable) GluR5 receptors are as sensitive to kainate-induced seizures as are control animals or animals expressing only unedited (calcium-permeable) GluR5 receptor subunits.[55] (Edited GluR5 kainate receptors are expected to have markedly reduced current densities.) Moreover, mice in which the GluR5 kainate receptor was deleted by gene targeting show identical sensitivity to systemic kainate as wild-type animals.[56] Thus, although GluR5 may be of special significance in the amygdala, GluR6 kainate receptors are most relevant to the overall convulsant activity of kainate.

How does activation of kainate receptors result in seizure generation? Depolarization induced by gating of these receptors (by either exogenous agonists or endogenously released glutamate) would be expected to directly excite amygdala neurons. However, this would not explain why kainate receptor agonists are uniquely capable of stimulating synchronized epileptiform discharges. Studies in the hippocampus indicate that kainate receptor activation has a variety of additional actions on inhibitory and excitatory systems that could contribute to seizure induction. For example, it has been shown that activation of kainate receptors can markedly enhance the spontaneous firing of GABAergic interneurons.[33,57] Although this might be expected to increase inhibition, extracellular GABA accumulation could desensitize postsynaptic $GABA_A$ receptors and activate presynaptic $GABA_B$ receptors, ultimately leading to reduced inhibition and the promotion of epileptic activity. Other studies have indicated that activation of kainate receptors on interneurons depresses GABA re-

lease.[21,58] In addition to effects on GABAergic inhibition, in the hippocampus, kainate receptors have complex modulatory effects on glutamate release. In most cases, this effect is inhibitory in nature and mediated through presynaptic mechanisms.[59] However, Contractor *et al.*[60] found that excitatory synaptic currents evoked in CA3 neurons by perforant path stimulation were enhanced by kainate receptor activation. Although it has yet to be determined whether this effect is mediated presynaptically on axon terminals or postsynaptically on dendrites, it failed to occur in slices from animals in which GluR5 or GluR6 had been deleted, indicating involvement of these kainate receptor subunits. Moreover, low concentrations of kainate can depolarize mossy fiber terminals, resulting in enhanced excitatory synaptic transmission onto CA3 pyramidal neurons,[61] and kainate receptors seem to play a role in forms of short-term synaptic facilitation at mossy fiber synapses,[62] although these effects largely occur through presynaptic mechanisms that require GluR6 and not GluR5 receptors. To the extent that such forms of synaptic facilitation are involved in the generation of epileptic activity, kainate receptor agonists with GluR6 activity (e.g., kainate but not ATPA) might act through these mechanisms.

In conclusion, despite the long-standing recognition of kainate receptors as a unique type of ionotropic glutamate receptor, the roles of these receptors in seizure generation and epileptogenesis are only now being defined, and the relative contributions of specific kainate receptor subtypes is still controversial. Kainate receptors containing the GluR6 subunit are undoubtedly important, given their widespread distribution and unique role in seizures induced by systemic kainate. Nevertheless, GluR5 appears to be of significance in the amygdala, a critical brain region for temporal lobe epilepsy. In the future, it is expected that kainate receptors will attract increasing attention as potential targets for the development of epilepsy drug treatments.

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TWENTY

Psychosocial Stressors as Predisposing Factors to Affective Illness and PTSD

Potential Neurobiological Mechanisms and Theoretical Implications

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SENSITIZATION IN THE AFFECTIVE DISORDERS

Stressor and Episode Sensitization in the Unmedicated State

At the beginning of the twentieth century, Kraepelin (1921) laid out the fundamentals of the sensitization hypothesis of affective disorders:

> the attacks begin not infrequently after the illness or death of near relatives ... we must regard all alleged injuries as possibly sparks for the discharge of individual attacks, but the real cause of the malady must be sought in *permanent internal changes*, which at least very often, perhaps always, are innate ... in spite of the removal of the discharging cause, the attack follows its independent development. But, finally, the appearance of wholly similar attacks on wholly dissimilar occasions or quite without external occasion shows that even there where there has been external influence, it must not be regarded as a necessary presupposition for the appearance of the attack. (pp. 180–181)

In this terse and insightful paragraph, he outlines four different components of the sensitization hypothesis: (1) initial episodes of affective illness are often precipitated by psychosocial stressors; (2) as recurrences emerge, later episodes do not require the same psychosocial precipitation, but may occur more spontaneously; (3) episodes tend to occur with a characteristic similarity; and (4) innate neurobiological mechanisms mediate these vulnerabilities and recurrences, and presumably these could occur both on an inherited and an experiential basis.

Other aspects of this sensitization hypothesis are outlined in additional passages from his work. Although Kraepelin noted the "sheer immeasurable multiplicity of clinical pictures," (p. 114) and "the frequency, with which the different clinical forms of

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manic-depressive insanity here described occur in a fairly large series of observations, is naturally very various . . ." (p. 133), ". . . for the most part the disease shows the tendency later on to run its course more quickly and to shorten the intervals, even to their complete cessation" (p. 137). "When the disease has lasted for some time and the attacks have been frequently repeated, the psychic changes usually become more distinct during the intervals also" (p. 149).

Thus, Kraepelin (1921) recognized both the inherent variability and unpredictability of bipolar episode presentation and course among and within individual patients; but within this seeming randomness, he noted a tendency for well intervals to decrease as a function of the successive number of episodes (i.e., sensitization) with a particularly striking effect after the first, second, and third episodes. Kraepelin recognized the poor prognostic implications of the occurrence of dysphoric mania and its high rate of hospitalization and chronicity, particularly in females. Herein he applied another postulate of the sensitization model, that with greater number of recurrences there may be a malignant progression and treatment resistance in the illness. We have summarized these essential elements of the sensitization hypothesis in Figure 20.1, with the more explicit hypothesis that greater numbers and/or faster cycling of episodes will be associated with greater treatment resistance, particularly to the classical modality of pharmacoprophylaxis – lithium carbonate.

Illness Progression During Tolerance Development

There is an additional component of the sensitization hypothesis in affective illness based on episodes breaking through previously effective pharmacoprophylaxis in a pattern that resembles tolerance. In these instances, patients who had previously been severely ill experience a good response to prophylactic monotherapy or combination therapy and remain well for a period of years, and then begin to experience breakthrough episodes of increasing severity or duration (Post et al., 1999; Post, Ketter, Denicoff, Leverich, & Mikalauskas, 1993; Post, Leverich, Rosoff, & Altshuler, 1990). These recurrences may progress to the point of complete loss of efficacy to what had previously been a highly effective treatment regimen.

A number of predictions in affective illness are based on a preclinical model of tolerance (Weiss, Clark, Rosen, Smith, & Post, 1995) to the anticonvulsant effects of mood stabilizing anticonvulsants on amygdala-kindled seizures (Table 20.1). These postulates, which remain to be further specifically examined for their clinical applicability, include:

1. A greater number of prior episodes (a marker of increased pathological illness drive) will be associated with a greater likelihood and more rapid onset of tolerance development;
2. Higher rather than minimally effective doses of a treatment will be more likely to prevent or delay tolerance development (for some, but not all drugs [e.g., with lamotrigine being a possible exception]);
3. Stable (or possibly descending) dose regimens will be preferable to minimally effective dosing followed by dose escalation in an attempt to treat breakthrough episodes;

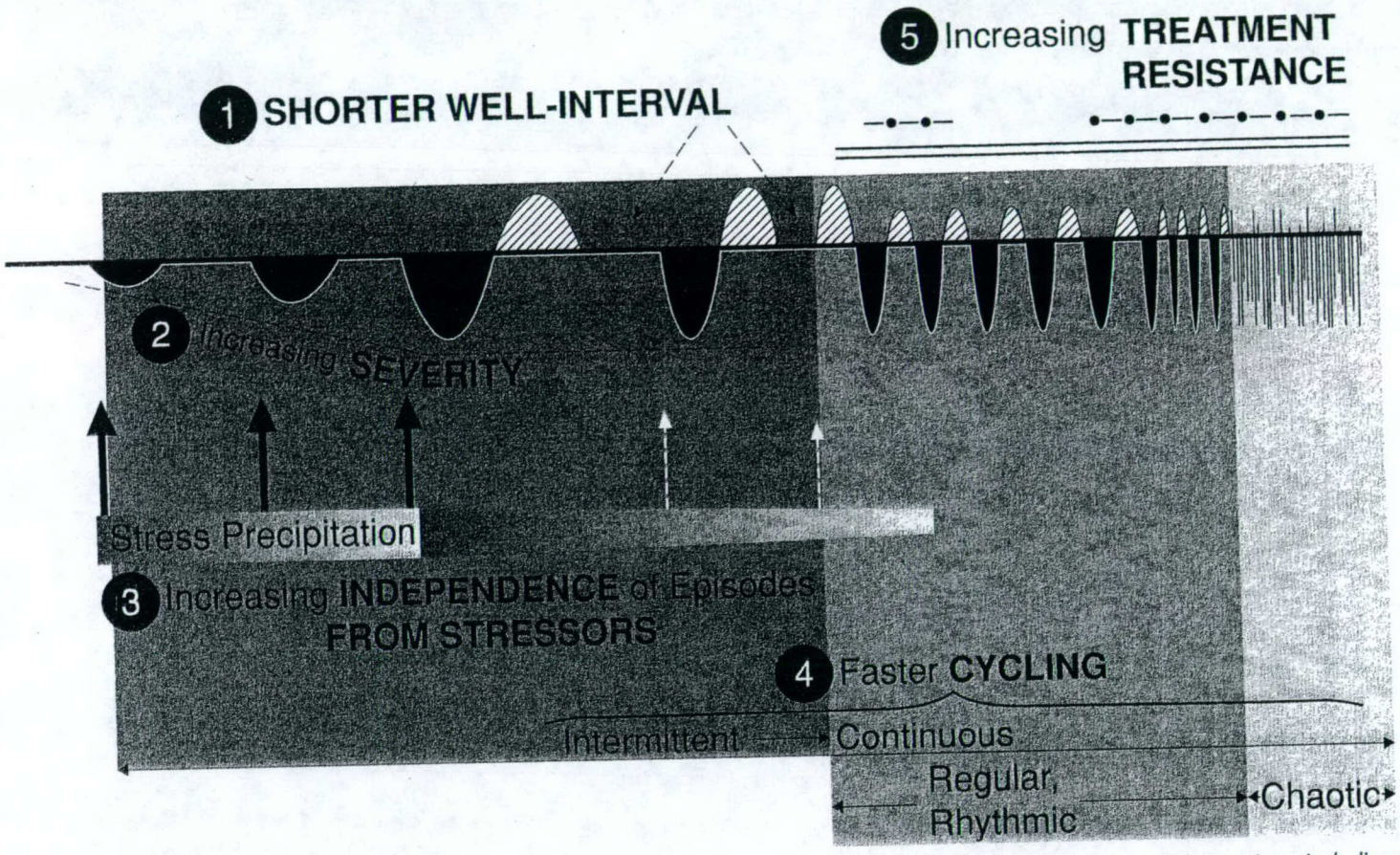


Figure 20.1. Sensitization in affective illness. Evidence of the tendency for the illness to progress is based on a variety of observations, including: (1) increases in episode frequency; (2) increases in episode severity, quality, or complexity; (3) early episodes precipitated by psychosocial stressors, but later ones occurring more spontaneously; (4) transition from intermittent to continuous to chaotic cycling patterns; and (5) possibly increasing treatment resistance, especially to lithium.

Table 20.1. Clinical Predictions[a] to Be Explored Based on Observations from a Preclinical Model of Amygdala-Kindled Seizures[b]

Tolerance to anticonvulsant effects slowed by:	Future studies; is there predictive validity for clinical tolerance in affective illness?
Higher doses (but lower doses w/LTG)	Maximum tolerated doses
Not escalating doses	Stable dosing
More efficacious drugs (VPA > CBZ)	Different rate of treatment resistance?
Treatments initiated early in illness	Sarantidis and Waters, 1981; Gelenberg et al., 1989; O'Connell et al., 1991; Denicoff et al., 1997; Swann et al., 1999
Combination treatment (CBZ plus VPA)	Combination > monotherapy?
Reducing illness drive	Treat comorbidities
Response restored by:	
Period of drug discontinuation then re-exposure	Randomized study of continuation treatment vs. discontinuation and re-exposure
Agents with different mechanisms of action (no cross-tolerance)	Cross tolerance from lamotrigine to CBZ, not VPA

[a] Right side of table.
[b] Left side of table.
VPA, valproic acid; CBZ, carbamazepine; LTG, lamotrigine.

4. Drugs with different mechanisms of action will have different potency in preventing tolerance development;
5. Combination treatments employing multiple and differential mechanisms of action will be more effective in delaying tolerance development than similar doses of several monotherapies;
6. When loss of efficacy via a tolerance mechanism has occurred, renewal of efficacy may be achieved by switching to or adding another drug with a different mechanism of action (i.e., one that does not show cross tolerance); and
7. In instances in which loss of efficacy has developed gradually via a purported tolerance mechanism, effectiveness of the initial drug may be re-achieved following a period of treatment with other agents and then reinstitution of the initial drug.

As noted below, some of these predictions based on the preclinical kindled seizure model have been preliminarily explored and partially validated, but a number remain to be directly tested in the clinic. Moreover, to the extent that these predictions of the model do prove valid, a third round of predictions could be derived based on presumed neurobiological and experiential effects on gene expression mechanisms involved. These also will be elaborated in a later section of the chapter.

Episode Sensitization

Kraepelin's empirical observations which form the basis of the sensitization model in affective illness have now been largely validated and confirmed by many investigators using a variety of study methodologies. Most studies support a greater role of

psychosocial stressors in initial, compared with later, episodes, with few exceptions (Table 20.2). Studies that have found that stressors continued to be associated with later episodes of affective illness have misinterpreted these observations as a refutation of the model (Hammen & Gitlin, 1997; Swendsen, Hammen, Heller, & Gitlin, 1995). This refutation is not true because the sensitization model is based on the assumption that over the course of illness, patients become *increasingly* sensitive to the role of stressors in the precipitation of episodes. To the extent that stressors are involved and documented, it supports the model; to the extent that later episodes are associated with or precipitated by symbolic stressors, conditioned stressors, or occur in the relative absence of exogenous stressors, this finding would also be consistent with the model. The model only suggests that there is a reduced need for the involvement of and direct triggering by stressors in later episodes.

Numerous studies support the general provision of cycle acceleration with shorter well intervals between successive episodes (Table 20.3), although, again, as Kraepelin and others have pointed out, there would be many individual exceptions to this rule. For example, we have observed a subgroup of patients who begin their illness with rapid or continuous cycling from the outset (Roy-Byrne, Post, Uhde, Porcu, & Davis, 1985) and, therefore, we would not expect any notable further degree of cycle acceleration; in fact, it might not be possible to demonstrate such a phenomenon in patients with continuous cycling from the outset because of a ceiling effect.

Moreover, Kraepelin (1921) made his observations in an era before major psychopharmacological interventions were available. Thus, many investigators who fail to observe this pattern or report that there is a lack of progression in the illness, or a failure of rapid cycling patients to continue in this pattern, have often not taken treatment into account (Coryell, Endicott, & Keller, 1992). Whenever effective treatment interventions are employed, the natural course of the illness would be altered, which is the goal of modern pharmacotherapeutic interventions. For example, Angst and Sellaro (2000) report that duration of the well interval only decreases over the first two episodes and then stabilizes thereafter.

Failure of well-treated patients to demonstrate this pattern of cycle acceleration and malignant progression of the illness cannot be taken as a refutation of the sensitization hypothesis. The fact that we and others have continued to observe this pattern of sensitization in treatment-refractory patients is predicated on their lack of adequate response to pharmacotherapy, presumably yielding a course and pattern of illness not entirely dissimilar from what might have been expected if they were untreated (as in the Kraepelinian era). It is also possible, however, that some treatment such as antidepressants could modify the course in an adverse fashion similar to that observed with levodopa treatment in Parkinson's disease, generating an increased rapidity of cycling of the on-off phenomenon (Nissenbaum et al., 1987).

The prediction that greater number of episodes prior to institution of lithium pharmacoprophylaxis is a negative prognosticator for response has also been widely replicated in the literature in two different types of studies. In the first type, studies overwhelmingly indicate that rapid cycling patients are less responsive to lithium than those without a rapid cycling pattern (Post, Ketter, Speer, Leverich, & Weiss, 2000; Post, Kramlinger, Altshuler, Ketter, & Denicoff, 1990). In addition, there is a considerable literature indicating that the number of episodes prior to institution of lithium

Table 20.2. Greater Association Between Life Events and First versus Subsequent Episodes of Affective Disorder

Author	Disorder	Number of Episodes	N	% Patients for Whom Major Life Events Preceded Episode: First Episode	% Patients for Whom Major Life Events Preceded Episode: Later Episode	p Value	Assessment
Matussek et al. (1965)	Depression	1	242	44		–	Stressors (138 psychologic; 58 somatic) had to clearly precede onset of episode
		2	135		34	–	
		3	82		24	–	
		4	119		19	–	
Angst (1966)	Depression	1	103	60		–	No inventory
		≥ 4			38	–	
Okuma and Shimoyama (1972)	Manic Depression	1	134	45		–	Any event (3 months prior)
		2	134		26	–	
		3	134		13	–	
Glassner et al. (1979)	Manic Depression	1	25	75		–	Event rated stressful by patient and on Holmes and Rahe Scale (1 year prior; usually 2–24 days); role loss critical in patients and comparison subjects
		≥ 1[a]			56		
Ambelas (1979)[b]	Mania	1	14	50		< 0.01	Paykel Life Events Scale (4 weeks prior); one-third of cases followed bereavement
		≥ 2		67	28		
Ayuso et al. (1981)	Depression	1	43	55.8		< 0.05	Social and somatic stressors; patients with late onset had more events than did those with early onset
		2	35		40.0		
		3	18		38.8		
		≥ 4	47		29.7		
Perris (1984)	Depression	1	37	62	50[c]	< 0.02	Semistructured interview; 56 item inventory (3 months prior)
		≥ 2	112	43	19[d]	< 0.001	

Dolan et al. (1985)	Depression	1	21	62		< 0.05	Bedford College-Life Events and Difficulties Schedule (6 months prior) (Brown, Harris, 1978)
		≥ 2	57		29		
Ezquiaga et al. (1987)	Depression	< 3	52	50		< 0.01	Semistructured interview (Brown, Harris); no effect on chronic stress
		≥ 3	45		16		
Ambelas (1987)	Mania	1	50	66		< 0.001	Paykel Life Events Scale (4 weeks prior)
		≥ 2	40		20		
Ghaziuddin et al. (1990)	Depression	1	33	91		< 0.05	Paykel Life Events Scale (6 months prior)
		≥ 2	40		50		
Cassano et al. (1989)	Depression	1	94	66.0		< 0.05	Paykel Life Events Scale
		≥ 2	173		49.4		
Hammen & Gitlin (1997)	Bipolar	0–8	52	40		0.05	More episodes, more stressors and relapsed faster
		≥ 9			76		
Castine et al. (1998)	Schizophrenia	≤ 3	32	more recent life events		0.01	Paykel Life Events Scale
Nierenberg et al. (1998)	Depression	1st vs. > 3 episodes	176	1st episode had more stressful negative life events compared with recurrent		0.037	Life Events Scale, Perceived Stress Scale

[a] For this group, the most recent hospitalization was preceded by a life event resulting in role loss.
[b] Of surgical comparison subjects, 6.6% had experienced recent major life events.
[c] Percentage for negative or undesirable events.
[d] Percentage for events involving psychological conflict.

Table 20.3. Early Studies of Life Course of Manic-Depressive Illness

Study	# of pts.	High UP/BP Pt. Ratio	Sex	Age of Onset (Peak or Mean Years)	Decreasing Well Interval	Observational Time (years) Retrospective (R) Prospective (P)	Late Age at Onset Predicts Increased Relapse	Comments
Swift (1907)	105	No	74 F 31 M		Yes	R	Yes	Study examined first episode in terms of prognosis. Prognosis is better if first episode is a depression than if it is mania.
Kraepelin (1921)	903	Yes	648 F 255 M	20–30	Yes	Variable up to a lifetime R,P	Yes	Study contained both a large number of patients and extended periods of observation. Few patients were observed for their complete life course.
Malzberg (1929)	11,393		6513 F 4880 M	mean = 40			Yes	Study examined only first admissions and reviewed relationship to duration of episode and recovery.
Paskind (1930)	633			21–30	Yes	R	Yes	Nonhospitalized patients studied – may be helpful as a comparison group to the hospitalized patients. *Late age of onset predicts increased duration of episodes.
Pollock (1931)	8438	No	519 F 3274 M	20–24		11 R	Yes	Study examined a large number of patients. Many unrecovered cases were discharged as improved. Age of onset between 20 and 40 years – better prognosis than patients younger or older.
Steen (1933)	493			20–40		8 R	Yes	Study confused other diagnostic groups (i.e., schizophrenia, schizoaffective) with BP patients. Twenty to 30-year age of onset predicts high rate of recovery.

Rennie (1942)	208	Yes	117 F 91 M	45–55		20 R		Seventy-nine percent of patients will have more than 1 episode during their lifetime; 50% will have less than 3 episodes; 93% (193) recovered from first episode; 21% (62) never had another recurrence.
Poort (1945)	141	No		20–30		10–15 R	No	Fifty percent (71) were recurrent UP or BP; 19% (27) went on to develop another type of psychosis such as schizophrenia, "hysteria," or sociopathy.
Lundqvist (1945)	319		196 F 123 M	< 30/mania > 50/depression	Yes	14–32 R	Yes	Patient population is difficult to assess because 28% (89) became chronic following their first episode and 7% (22) developed schizophrenia. First episode of mania predicts increased risk for relapse.
Stenstedt (1952)	216	Yes	126 F 90 M	mean = 38.7 F		29 R		Study does not detail polarity of episodes in UP and manics who relapse; 11.7% was the morbidity risk of the illness among siblings and children of probands; 83% (117) had first episode as a depression; 53% (114) had one episode.
Astrup et al. (1959)	270	Yes				5–19 R	No	Study separates schizoaffective from manic-depressive illness; emphasizes the need for long-term follow-up to make separation

(continued)

Table 20.3 (*continued*)

Study	# of pts.	High UP/BP Pt. Ratio	Sex	Age of Onset (Peak or Mean Years)	Decreasing Well Interval	Observational Time (years) Retrospective (R) Prospective (P)	Late Age at Onset Predicts Increased Relapse	Comments
Angst and Weiss (1967)	388	Yes		mean = 38.5 (BP)	Yes	7 R	Yes	Study clarifies set of definitions for episode, interval, cycle. Confirms by use of statistics the earlier observations of the relationship of age of onset and number of episodes with prognosis. Only 12% (45) of patients were BP type.
Bratfos and Haug (1968)	207	Yes	116 F 91 M	mean = 35	Yes	6 P	No	Patients had various types of somatic therapy (ECT, antidepressant, and neuroleptics). Study did not distinguish between the type of therapy received in terms of risk for relapse; 20% (41) remained chronically ill.
Perris (1968)	270	No	144 F 126 M	mean = 37.7		20 R		Eighty-four percent of UP patients will convert to BP illness before 3 episodes, i.e., 16% of BP patients will be misdiagnosed as UP with up to 3 observations of depressive episodes. BP patients are at higher risk for relapse than UP.
Grof et al. (1974)	987	Yes			Yes	Up to 45	Yes	Patients were treated only during the acute phases of their illness and not prophylactically. Each succeeding cycle length is shorter on the average than the preceding one.

Taschev (1974)	652	Yes	350 F 323 M		Yes	R	Yes	Retrospective evaluation of cyclothymic depression (122, 18.7%), recurrent depression (134, 20.5%), involutional depression (335, 51.3%), reactive depression (23, 3.5%), recurrent mania (38, 5.8%); 26.4% (172) of depressives committed suicide; 16% (104) became chronic; no mention if patients were treated.
Angst et al. (1978)	254	Yes				12–16 P		Study of the number of episodes before conversion of UP or BP. Conversion of UP to BP > 3 episodes = 70%; > 6 episodes = 83%. Conversion of BP to schizoaffective: 3 of 40 (7.5%).
Angst (1978)	95	No	58 F 37 M	mean = 61		26 R, P		Statistical description of long-term observation of BP illness. Study examines the heterogeneity of BP illness. Females exhibit more depression than mania. Males exhibit a symmetrical distribution of mania/depression.
Zis et al. (1980)	334	Yes			Yes		Yes	Increased risk of relapse is a function of number of previous episodes.
Maj et al. (1992)	72	Yes	42 F 30 M	mean = 32.6	Yes	P	No	Increasing severity from the index episode to the first, second, and third prospective episodes.
Angst and Sellaro (2000)	549	No			Yes	329 R 220 P	No	Increased rapidity of episode recurrence only after the first 2 episodes.

Key: UP = Unipolar; BP = Biploar; ECT = Electroconvulsive Therapy.

Table 20.4. More Episodes Prior to Starting Lithium Is Associated with Poor Prophylactic Response

Investigator	Correlates of Poor Response to Lithium
Prien et al., 1974	High frequency of hospitalizations
Sarantidis and Waters, 1981	More episodes per year
Abou-Saleh and Coppen, 1986	Higher number of episodes (7.0 ± 1.3) in bipolar patients
Gelenberg et al., 1989	> 3 prior episodes
O'Connell et al., 1991	≥ 3.8 mean episodes
Goldberg et al., 1996	≥ 2 prior hospitalizations
Denicoff et al., 1997	• Older age at first treatment • Longer duration of illness • More than 1 hospitalization for mania
Maj et al., 1998	≥ 7.2 mean episodes
Swann et al., 1999	≥ 10 prior episodes

prophylaxis is a negative predictor of lithium response (Table 20.4). Of course, it is also possible in these uncontrolled studies that a greater number of episodes is only a marker for a subsequent adverse course that would have been manifest even if lithium prophylaxis were instituted after the first or second episode.

Although the study of Kessing, Bolwig, and colleagues (1998) involving more than 20,000 patients in the Danish Case Registry did not initially control for treatment, it is striking that they found a direct relationship between the number of prior episodes and both the incidence of and latency to relapse into another episode in unipolar and bipolar patients. These data from a country in which lithium is very widely used provide some of the strongest evidence of an overall sensitization effect, that is, greater number of prior episodes is associated with a greater risk of relapse in both of these affective disorders. This trend apparently emerges irrespective of whether or not patients were treated in the community with sustained prophylactic medication (although the direct analysis of this inference remains to be reported).

Stressor and Episode Sensitization

In another seminal study, in more than 600 female identical twin pairs, Kendler et al. (1993) showed that strong predictors of major depression included both stressors and number of prior episodes. They also documented that a variety of early life stressors such as lack of parental warmth and parental loss were associated with the onset of initial or minor (neurotic) depressions and that more concurrent psychosocial stressors were involved in the precipitation of recurrent episodes. Thus, these data appear to support an early vulnerability factor of the environment, perhaps leaving the patient at a higher risk for subsequent stressor-related induction of depression. Kendler et al. also found that neurotic or minor depression predisposed to major depression and prior major depressive episodes predisposed to further depressions. A genetic component accounted for part of the variance in both the initial and recurrent episodes.

Thus, there is substantial evidence for two types of sensitization: (1) most prominently, early childhood and subsequent adult life stressors can act as precipitants or vulnerability factors (stressor sensitization), and (2) episode sensitization, in which the number of prior episodes correlates with the likelihood of relapse and a shortening of

Table 20.5. Sensitization Phenomena in Affective Illness

Observation	Evidence	Investigators
↑ FREQUENCY OF RECURRENCE		
Well interval	+++	Kraepelin; Grof; Post
↑ SEVERITY		
As function of episode number	+	Maj
↑ CHRONICITY		
Each new episode carries added 10% risk of nonrecovery	++	Thase
↑ TREATMENT RESISTANCE		
First major depression more responsive than second	+	Angst
Greater number and frequency of prior episodes associated with lithium nonresponse	+	Gelenberg; O'Connell; Denicoff
↑ ABNORMAL NEUROBIOLOGY		
More Episodes:		
– More sleep abnormalities	++	Armitage; Thase
– Greater hypercortisolism	+	Ribeiro; Gurguis

the well interval. Preliminary data (Table 20.4) also suggest greater treatment refractoriness as a function of number of prior episodes.

Given these two major perspectives of the model (i.e., stressor and episode sensitization), it is then necessary to ascertain which underlying neurobiological mechanisms could mediate such long-term vulnerability from early stressors and episodes of affective illness themselves. Kraepelin (1921) wrote "... the real, the deeper cause of the malady is to be sought in a permanent morbid state which must also continue to exist in the intervals between the attacks" (p. 117). Although there are only a modicum of data available on the neurobiological correlates of episode sensitization in man, as outlined in Table 20.5, there is a very considerable preclinical literature that at least provides a basis for examining their potential relevance to the clinic.

There is considerable evidence for cross-sensitization between some types of stressors and cocaine administration, suggesting that elements of cocaine sensitization may parallel phenomena observed in stressor sensitization (Antelman, Eichler, Black, & Kocan, 1980; Kalivas & Duffy, 1989; Post, Ketter, Speer, Leverich, & Weiss, 2000). However, episodes of cocaine-induced hyperactivity and stereotypy can, in addition, serve as models of brief episodes of manic-like hyperactivity and frantic psychomotor drive that are not unlike those that occur in some patients with mania and dysphoric mania, respectively. As such, cocaine sensitization may be examined from the perspective that it could model aspects of both stressor sensitization and manic episode sensitization, and direct one toward the examination of whether some parallel neurobiological mechanisms are involved in all three types.

Sensitization and Kindling Phenomena Differences

We will only briefly allude to the neurobiology of another model of long-lasting neuronal learning and memory – amygdala kindling, which differs considerably (in behavior, biochemistry, neural pathways, and pharmacology involved) from sensitization

(Weiss & Post, 1994). The kindling model is an interesting model not only for examining the development of epileptogenesis, but also for observing lasting changes in neural and behavioral responsivity in response to very brief periods of brain stimulation. In amygdala kindling, repeated administration of subthreshold amygdala stimulation evokes afterdischarges of increasing frequency, duration, and complexity which then spread throughout the brain, involving other limbic and cortical structures in association with the development of increasingly robust and complex behavioral phenomena, culminating in a full-blown generalized seizure (Goddard, McIntyre, & Leech, 1969; Racine, 1972a, 1972b). Following sufficient numbers of amygdala stimulations associated with completed kindled seizures, a phase of spontaneity may emerge in which animals exhibit true epilepsy and have spontaneous seizures in the absence of exogenous physiological stimulation.

Thus, the kindling model provides a readily identifiable set of physiological and behavioral concomitants of neuronal learning and memory, each of which demonstrates an obvious augmented response to repeated brain stimulation and then culminates in a further progression to spontaneous episodes. At the level of both physiology (amygdala excitability thresholds and afterdischarges as well as their spread to other brain areas) and behavior (seizure stage evolution from partial to full-blown and then to spontaneous), increases in responsivity occur.

Although these electrophysiological and behavioral progressions present clear evidence for neuronal learning and memory phenomena, it is equally clear that they do not represent endpoints directly parallel to those that occur in bipolar illness (Weiss & Post, 1994). Complex partial seizures of the temporal lobe are often associated with prominent affective symptoms, but, conversely, primary affective illness is rarely associated with seizure-like manifestations. Moreover, the induced seizures of electroconvulsive therapy are used as a prominent treatment for severe manic and depressive episodes. Thus, kindling to a seizure endpoint must be considered a nonhomologous model for the affective disorders, since neither the inducing phenomena, nor the behaviors or their temporal domains, nor the pharmacological interventions between the two are identical or even highly similar.

However, kindling may be useful in understanding how a complex behavioral phenomenon such as a major motor seizure comes to be evoked by previously subthreshold stimuli upon repetition and, as well, how such precipitated episodes may proceed to the spontaneous variety. In addition, the kindled seizure model is particularly appropriate for examining tolerance development to the anticonvulsant effects of a variety of effective antiepileptic agents (Weiss, Clark, Rosen, Smith, & Post, 1995) many of which are now also used for the treatment of affective disorders (Dunn et al., 1998; Post et al., 1996, 1998a, 1998b). One can examine the principles and underlying neural mechanisms of tolerance development to these agents in the seizure realm and ask whether or not similar phenomena exist for tolerance development in other models or clinical situations, such as the tolerance that can occur in the prevention of paroxysmal pain syndromes (Pazzaglia & Post, 1992), migraine headaches (Post & Silberstein, 1994), breakthrough panic attacks, and most pertinent to the current discussion, episodes of recurrent affective disorder (Post, Ketter, Denicoff, Leverich, & Mikalauskas, 1993; Post & Weiss, 1996).

Thus, the stressor and cocaine sensitization models have direct parallels and homologies to phenomena that occur in the clinical realm in the course of unipolar and

bipolar affective disorders, whereas kindling must be considered only as an analogy and used for its indirect parallels. The kindling model's degree of predictive validity in this indirect realm remains to be determined, but will in turn reveal its ultimate utility.

NEUROBIOLOGICAL MECHANISMS FOR LONG-LASTING BEHAVIORAL AND BIOCHEMICAL VULNERABILITIES FOLLOWING EARLY LIFE STRESSORS

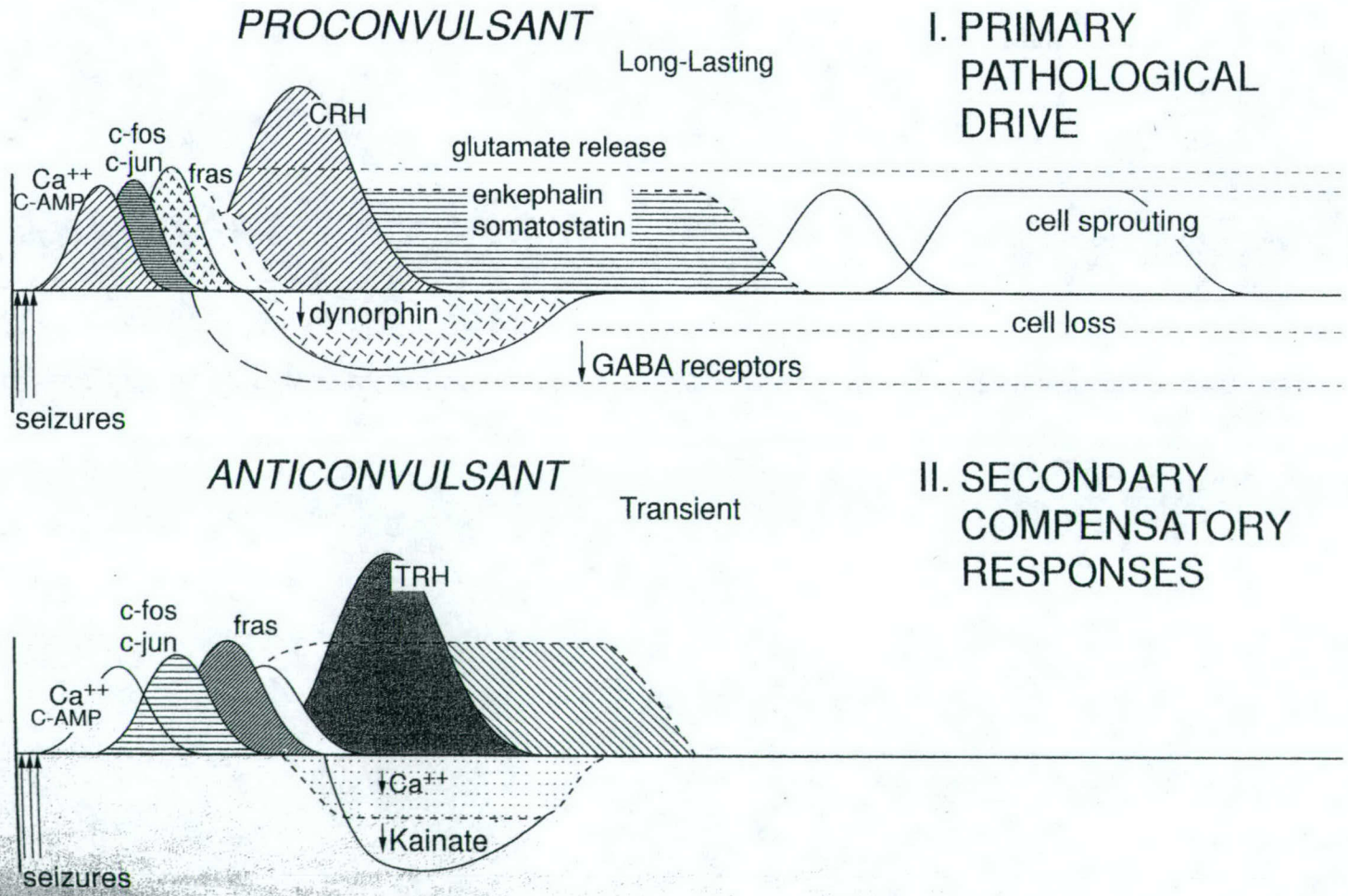
Levine et al. (1991) have used a paradigm of a single 24-hour period of maternal deprivation in rat pups as an inducer of long-lasting altered behavior and biochemical responsivity. These animals, like those of Plotsky and colleagues (Francis, Caldji, Champagne, Plotsky, & Meaney, 1999; Ladd et al., 2000; Plotsky, 1997) which are subjected to repeated episodes of three hours of maternal deprivation in the first weeks of life, show long-lasting hypercortisolism and increased anxiety-like behaviors.

Neurobiology of Repeated Maternal Separation: Parallels to the GR Knock-out Mouse

The studies of Plotsky and Meaney are particularly interesting from the perspective of adaptive and homeostatic mechanisms, because animals subjected to only fifteen minutes of daily maternal separation are protected against age-related loss of hippocampal anatomy and associated decline in learning and memory skills (Anisman, Zaharia, Meaney, & Merali, 1998; Liu et al., 1997; Meaney, Aitken, Van Berkel, Bhatnagar, & Sapolsky, 1988). Meaney has demonstrated that the mechanism of this effect is an increase in maternal licking behavior that occurs following the fifteen-minute separation, but not after a three-hour separation. After three hours, the previously separated pups are apparently not well identified and maternal behavior is degraded, with increased agitation in the mother and, in some instances, apparent frantic trampling of her offspring. It appears that this element of maternal behavior and neglect is a crucial element in producing the long-lasting hypercortisolism and anxiety-like behaviors in the separated offspring. This behavior can be remedied or prevented if the mother is given substitute rat pups during the three-hour period of separation from her own pups. In this case, when the separated pups are returned, the mother's behavior is normal and no lasting behavioral or biochemical alterations in the offspring are produced (Meaney, 1999).

In the three-hour separated pup that receives the full separation/maternal malbehavior, it is remarkable that these animals as adults show an increased proclivity to self-administer alcohol and cocaine compared with litter mates without such early stressors (Meaney, Brake, & Gratton, 2002; Huot, Thrivikraman, Meaney, & Plotsky, 2000). The entire biobehavioral syndrome, including hypercortisolism, is reversed by serotonin-selective antidepressants. However, if the antidepressant drug treatment is discontinued, animals revert to their prior anxious and hypercortisolemic state.

This model thus provides a dramatic illustration of how experiences in the environment can induce lasting behavioral and neurobiological changes at the level of gene expression. Increases in corticotropin-releasing factor (CRF) mRNA, for example, have recently been demonstrated in the hypothalamus of these animals (Francis, Caldji, Champagne, Plotsky, & Meaney, 1999). Moreover, the ability of these early stressors to alter the expression of these behavioral and biochemical changes in a life-long fashion parallels a related syndrome that can be induced genetically using transgenic animals.


PROCONVULSANT
Long-Lasting
I. PRIMARY PATHOLOGICAL DRIVE
CRH
c-fos
c-jun
fras
Ca++
C-AMP
glutamate release
enkephalin
somatostatin
cell sprouting
dynorphin
cell loss
GABA receptors
seizures
ANTICONVULSANT
Transient
II. SECONDARY COMPENSATORY RESPONSES
TRH
c-fos
c-jun
fras
Ca++
C-AMP
Ca++
Kainate
seizures

its putative role in such a relationship. Supporting the possibility that BDNF could be a crucial factor in learning and memory defects are the observations of Korte et al. (1998) indicating that transgenic animals with an absence of BDNF have both deficient long-term potentiation (LTP), and an inability to navigate accurately in the Morris maze test, indicating a deficit in the ability to perform normal tasks that are ordinarily well within the animal's normal repertoire.

One is now in a position to ask questions such as what are the crucial degrees of BDNF, CaMKII, or iNOS decrement that might be etiopathological to the observed behavioral and biochemical alterations. We would imagine that there is a considerable range of different parameters that may influence the ultimate impact and outcome of a stressor, including severity, duration, quality, and timing, as well as the number of repetitions and recurrences later in development. The degree of both genetic vulnerability and stressor resistance or resilience (Luthar, Cicchetti, & Becker, 2000) in conjunction with the potential for adaptation to the stressor and the support provided by others (Breier et al., 1988) may all be crucial variables in whether long-term pathological neurochemical and behavioral alterations become manifest.

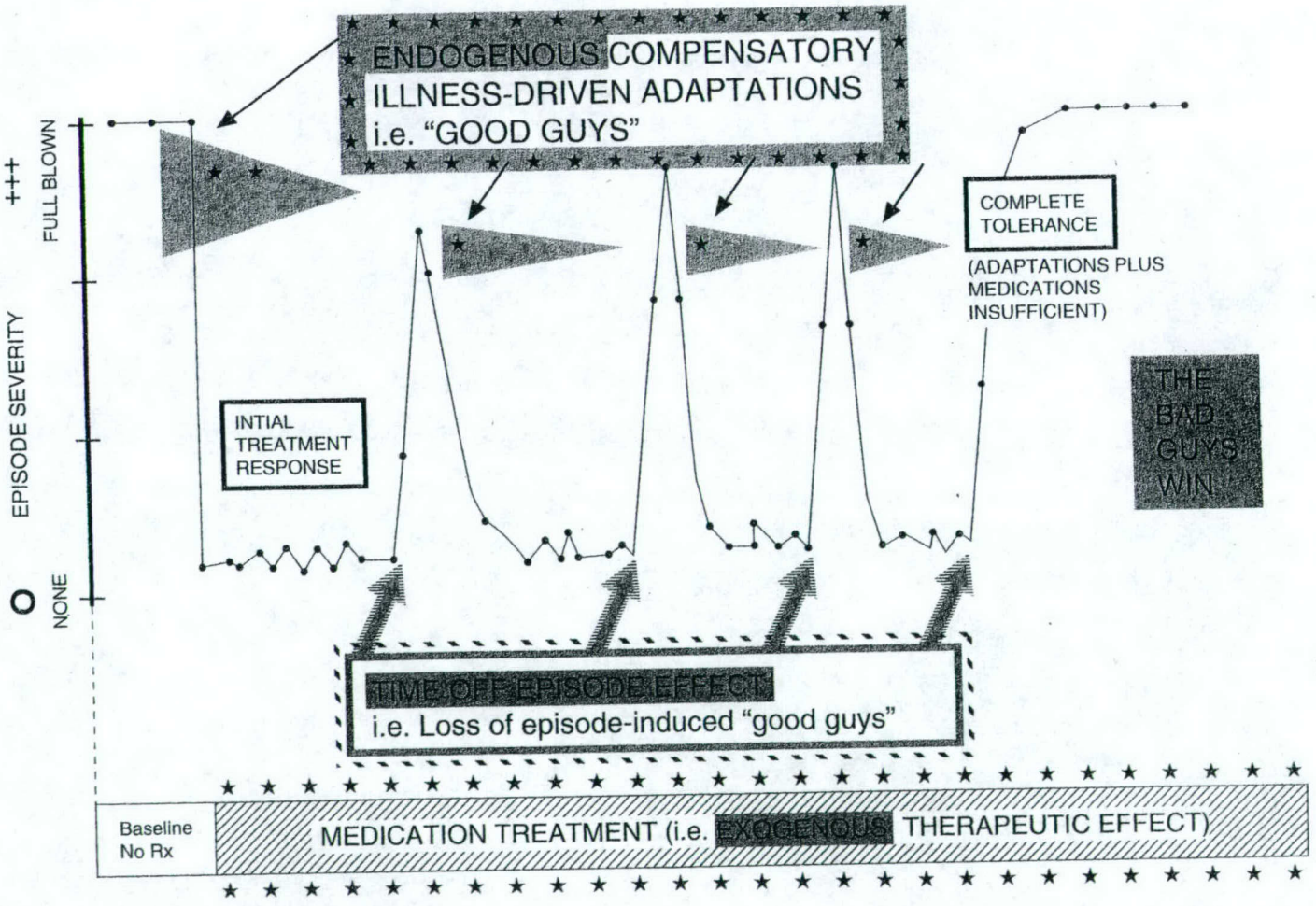
Pathological versus Adaptive Alterations in Gene Expression in Kindled Seizures

It is perhaps useful to discriminate between changes in gene expression that are related to the primary pathology of sensitization versus those that are compensatory and adaptive. A similar distinction is more readily identifiable for amygdala-kindled seizures. Here we have provisionally divided the many changes in gene expression into these two components (Figure 20.3). One is based on whether they are primary and important to the maintenance of the kindled "memory" trace, or whether they reflect endogenous anticonvulsant adaptations that attempt to return the animal to homeostasis (Post & Weiss, 1992, 1996).

This separation has potential clinical importance from a number of perspectives, perhaps the most significant of which is that it provides new, dual, and differential targets of therapeutics. One can attempt to both block the primary pathological changes of kindling progression and, conversely, enhance the endogenous anticonvulsant or adaptive processes.

We have preliminarily identified thyrotropin-releasing hormone (TRH) as an endogenous anticonvulsant substance because it transiently increases in the hippocampus after seizures and has been reported to be anticonvulsant (Kubek, Liang, Byrd, & Domb, 1998). We have extended these observations with the finding that intra-hippocampal injection of TRH suppresses amygdala-kindled seizures (Wan, Noguera, & Weiss, 1998).

Figure 20.3. Schematic illustration of potential genomic, neurotransmitter, and peptidergic alterations that follow repeated kindled seizures. Putative mechanisms related to the primary pathological drive (i.e., kindled seizure evolution) are illustrated on top and those thought to be related to the secondary compensatory responses (i.e., anticonvulsant effects) are shown on the bottom. The horizontal line represents time. Sequential transient increases in second messengers and immediate early genes (IEGs) are followed by longer lasting alterations in peptides, neurotransmitters, and receptors or their mRNAs, as illustrated above the line, whereas decreases are shown below the line. Given the potential unfolding of these competing mechanisms in the evolution of seizure disorders, the question arises regarding whether parallel opposing processes also occur in the course of affective illness of other psychiatric disorders. Endogenous adaptive changes (bottom) may be exploited in the design of the new treatment strategies.


ENDOGENOUS COMPENSATORY
ILLNESS-DRIVEN ADAPTATIONS
i.e. "GOOD GUYS"
COMPLETE
TOLERANCE
(ADAPTATIONS PLUS
MEDICATIONS
INSUFFICIENT)
THE
BAD
GUYS
WIN
INTIAL
TREATMENT
RESPONSE
+++
FULL BLOWN
EPISODE SEVERITY
O
NONE
TIME OFF EPISODE EFFECT
i.e. Loss of episode-induced "good guys"
Baseline
No Rx
MEDICATION TREATMENT (i.e. EXOGENOUS THERAPEUTIC EFFECT)

This is of additional interest because, in animals that have become tolerant to the anticonvulsant effects of either carbamazepine or diazepam, seizures that normally increase TRH mRNA no longer do so (Weiss, Clark, Rosen, Smith, & Post, 1995). It is postulated that the failure to induce some compensatory adaptive changes in gene expression (such as TRH, $GABA_A$ receptors, etc.) may be intimately involved with the tolerance process. This would also provide a conceptual mechanism for how the therapeutic efficacy of a drug may be revived after tolerance has developed following a period of time off the drug (which would theoretically allow seizures to again induce the TRH mRNA and other adaptive changes in gene expression) and thus facilitate carbamazepine's effectiveness.

Pathological versus Adaptive Changes in Gene Expression in Affective Disorders?

Not only have we postulated that the relative balance and predominance of primary pathological compared with the secondary and adaptive alterations in gene expression are associated with the development of loss of efficacy to some classes of anticonvulsants in the tolerance model, but this ratio might also be relevant in determining, in the medication-free state as well, whether seizures are manifest or not during kindling development and expression. Do parallels exist in the progressive evolution of illness in other syndromes? In this regard, we would surmise that the increases in CRF (in turn driving increases in cortisol) may be representative of one of the primary pathological processes of depression evolution and progression, particularly since hypercortisolemia has been associated with depression and cognitive impairment in other syndromes, such as Cushing's disease (Starkman, 1993).

Conversely, the increases in TRH directly reported in cerebrospinal fluid (CSF; Banki, Bissette, Arato, & Nemeroff, 1988) or inferred from the associated blunting of TSH response to TRH (suggestive of subclinical hyperthyroidism; Loosen, 1985), may be part of an endogenous set of antidepressant mechanisms that help to naturally terminate a depressive episode. Thus, as schematized in Figure 20.3, we again hypothesize that it is the ratio of the primary pathological versus secondary adaptive changes in gene expression that determine whether an individual remains in a relatively euthymic state or proceeds toward the recurrence of further episodes of affective disorder (Figure 20.4).

Figure 20.4. Hypothetical schema of the role of endogenous regulatory factors in the generation and progression of illness cyclicity. After an illness episode, adaptive compensatory mechanisms are induced (i.e., "good guys"; shaded triangle with two stars), which together with drug treatment suppress the illness (initial treatment response; box). The "good guys" dissipate with time (i.e., the time-off seizure effect), and episodes of illness re-emerge. Although this re-elicits illness-related compensatory mechanisms, the concurrent drug treatment prevents some of the illness-induced adaptive responses from occurring (smaller triangles with one star). As tolerance proceeds (associated with the loss of adaptive mechanisms), faster illness re-emergence occurs. Thus, the drug is becoming less effective in the face of less robust compensatory mechanisms. The primary pathology is progressively re-emerging, driven both by additional stimulations and episodes (i.e., the kindled memory trace, or the "bad guys") along with a loss of illness-induced adaptations. Because this cyclic process is presumably driven by the ratio of the "bad vs. good guys" at the level of changes in gene expression, we postulate that such fluctuations in the "battle of the oncogenes" arising out of illness and treatment-related variables could account for individual patterns in illness cyclicity.

Table 20.6. Incidence of Traumatic Stressors in Bipolar Patients in the Stanley Foundation Bipolar Network

	Child *N*(%)	Adolescent *N*(%)	Adult *N*(%)
Physical Abuse			
Not Abused	228 (76)	242 (80)	246 (88)
Abused	71 (24)	59 (20)	37 (12)
Sexual Abuse			
Not Abused	241 (76)	247 (83)	242 (82)
Abused	59 (20)	51 (17)	55 (18)

IMPACT OF EARLY STRESSFUL EXPERIENCES IN BIPOLAR AFFECTIVE DISORDER

Although a number of studies outlined in Table 20.2 have examined the relative role of stressors in initial versus later episodes of both unipolar and bipolar depression, there has been less examination of the impact of early stressful life events on the subsequent course of bipolar illness. In the Stanley Foundation Bipolar Treatment Outcome Network, which now follows more than 500 patients on a detailed daily basis with the NIMH-LCMp (prospective version of the life chart method) (Leverich et al., 2001), we had the opportunity to address this question in 631 consecutive outpatients who completed a detailed questionnaire which included items related to whether or not they were exposed to physical or sexual abuse in childhood or adolescence (Leverich et al., 2002).

The incidence rate of these types of extraordinary psychosocial stressors listed in Table 20.6 is parenthetically and disappointingly not that different from the rate observed in many unselected nonclinical populations. However, in the context of patients with bipolar illness, the reported occurrence of either early physical or sexual abuse in childhood or adolescence was highly associated with an earlier onset of affective illness and more rapid, ultrarapid, and ultradian cycling patterns (Table 20.7). In the univariate analyses subjected to Bonferroni correction and in the logistic regression analysis, other

Table 20.7. Type of Early Abuse (Childhood or Adolescence) and Characteristics of Early Abuse

	Physical Abuse *N*(%)	Sexual Abuse *N*(%)
Early Onset	92 (50)***	96 (50)***
Ultradian Cycling	52 (41)**	43 (34)
Increased Severity of Mania	116 (63)***	106 (56)**
Increased Severity of Depression	119 (64)	123 (64)**
Attempted Suicide	90 (49)***	90 (48)***

*** $p < .001$
** $p < .01$
* $p < .05$

variables remain significant as well. Physical abuse included self-reports of a pattern of increasing severity of mania and a family history of bipolar disorder, alcoholism, drug abuse, or other psychiatric illnesses. Sexual abuse included self-reports of an increased incidence of attempted suicide (45%) and a family history of drug abuse and other psychiatric illnesses.

There was also an increase in the number of clinician-rated Axis I lifetime comorbidities (2.0) in those experiencing these early traumatic life experiences versus those without these early stressors (1.3, $p < 0.002$). Physical abuse was associated with a significant increase in anxiety disorder, drug abuse, alcoholism, and a diagnosis of PTSD. Sexual abuse was selectively associated with a lifetime history of drug abuse. Those with early stressor history also had greater numbers of Axis II comorbidities. Physical abuse had a strong association with increased presence of cluster A disorders (i.e., the odd, eccentric) including paranoid, schizoid, and schizotypical disorders. Sexual abuse was most strongly associated with the presence of cluster B disorders (i.e., dramatic, emotional, including histrionic, narcissistic, borderline, and antisocial disorders).

Moreover, in addition to the retrospective self-reported illness variables related to earlier onset and greater severity in the unfolding of bipolar illness (mania and suicidality), in the prospective year of clinician ratings we observed that those with a history of physical and/or sexual abuse in childhood and adolescence were more ill than those without such a history (Leverich et al., 2002). This was revealed in both an increased percentage of time well measured on the LCM as well as on the increased levels of depression severity measured on the Inventory of Depressive Symptomatology (IDS) (Rush et al., 1986, 1996).

CLINICAL APPROACHES TO BIPOLAR ILLNESS AND ITS PREVENTION

Caveats

The causal relationships in the data described above are not easily discerned or readily disentangled. Although it is highly plausible to first think that these early life experiences could lead to altered neurochemistry through some of the mechanisms described in the previous preclinical sections, and thus change the likelihood and severity of bipolar symptom development and evolution, it is also possible that traits associated with increased severity of later illness could evoke increased physical or sexual abuse. Lastly, it is possible that another or third variable, such as genetic loading, could determine both the more severe pattern of illness and the tendency for increased physical or sexual abuse (either evoked or directly related to parental illness), rather than any direct causal relationship between early abuse and more severe course of illness characteristics.

Notwithstanding these causal ambiguities, the strong relationships suggest the importance of attempts at earlier intervention. Those who were physically or sexually abused had a longer period of time from first affective symptoms to first treatment (13 ± 11 years) than those who were not abused (8 ± 9 years; $p = .0003$). Even eight years in the nonabused group is far too long; the average treatment delay in many populations is about ten years, including the Stanley Network (Suppes et al., 2001), the surveyed members of the National DMDA (Lish, Dime-Meenan, Whybrow, Price, & Hirschfeld, 1994), or other clinical research cohorts (Egeland, Hostetter, Pauls, & Sussex, 2000).

Early Intervention

Thus, there is a great need for earlier recognition and initiation of treatment in patients with bipolar illness in general. It would appear even more critical for those at high risk for more severe illness progression and negative prospective outcomes based on high genetic loading or the occurrence of early stressors. Yet it is just these adolescents and adults who are likely to have the longest delays in beginning treatment.

In addition to helping prevent serious affective dysfunction, earlier intervention may help a child avoid the variety of Axis I (McElroy et al., 2001), Axis II (Leverich et al., 2000), and medical comorbidities that are associated with these early stressful life experiences. In particular, the increased rate of substance abuse is already a problem in general in bipolar illness (Regier et al., 1990), and now we have found there is an additional greater risk for those with these earlier adverse life experiences. Forty-eight percent of those with a history of sexual abuse versus 19 percent without ($p = 0.00025$) have a lifetime diagnosis of drug abuse in the Stanley Foundation Bipolar Network; 40 percent of those with a history of physical abuse compared with 22 percent without have a history of drug abuse, and 52 percent versus 31 percent have a history of alcohol abuse ($p = 0.025$) (Leverich et al., 2000).

Thus, it would appear prudent to recommend primary substance abuse prevention techniques in the child and adolescent with bipolar illness, particularly in the presence of a history of physical and/or sexual abuse in childhood and/or adolescence. One can only wonder about the potential parallels of these vulnerabilities to the findings of increased alcohol and cocaine self-administration in the adult rodents that had previously experienced repeated maternal separation as pups (Meaney et al., 2002; Huot et al., 2002).

The association between stressful episodes and precipitation of the first episode of illness, as well as the current episode of illness in those with a history of early physical or sexual abuse compared with those without, also provides an important area for pharmacotherapeutic and psychotherapeutic intervention. To the extent that these individuals are particularly vulnerable to stressor precipitation of episodes and/or at increased risk of exposure to more stressors in general (Table 20.8), as our data would suggest ($p < 0.0001$ for physical abuse and $p < 0.001$ for first sexual abuse [Leverich et al., 2002]), dealing with this likelihood on a direct basis with appropriate cognitive, behavioral, interpersonal, or other focused psychotherapeutic techniques, as well as pharmacotherapeutics, may be of great assistance in raising the threshold for episode precipitation. Putting coping strategies and alternative perspectives into place that would enable the individual to be more comfortable in the face of stressor occurrence may be particularly valuable. The great potential for therapeutic benefit of these types of interventions are delineated in Cicchetti, Rogosch, and Toth (2000) and Luthar, Cicchetti, and Becker (2000).

Opposite Effects of Stress and Psychotrophic Drugs on Gene Expression and Neurogenesis

New data suggest additional theoretical rationales for psychotherapy besides providing additional psychosocial support based on the general therapeutic relationship and specialized techniques employed. To the extent that therapy and the development of

Table 20.8. Incidence of Stressful Life Events prior to First Episode and Most Recent Episode in Patients with or without a History of Early Abuse (Data from Leverich et al., 2002)

	Prior to First Episode	Prior to Most Recent Episode
History of Early Physical Abuse		
Absent	2.5	2.8
	]***	]***
Present	4.2	4.8
History of Early Sexual Abuse		
Absent	2.5	2.8
	]***	]***
Present	3.9	4.4

*** = $p < 0.001$

coping strategies can lessen the impact of stressors, they could ultimately lessen the effects of stressors on gene expression. Although this remains only a theoretical possibility, it has been demonstrated that antidepressant compounds have a variety of effects on neurotrophic factor gene expression that are opposite to those of stress (Duman, 1998; Smith et al., 1995). For example, stress depletes BDNF in the hippocampus while chronic antidepressant treatment increases BDNF in this area. Moreover, both Smith and colleagues in our laboratory (Smith et al., 1995) and Duman (1998) have demonstrated that pretreatment with antidepressants may block some or all of the associated effects of stress on neurotrophic factor gene expression. Most recently, this paradigm has been extended by Gould and Tanapat (1999) and others, indicating that the antidepressants (and lithium) increase neurogenesis even in the adult animal, whereas stressors produce the opposite effect (Table 20.9).

Table 20.9. Impact of Stress and Psychotropic Drugs on Gene Expression and Brain Structure

	STRESS	Gluco-corticoids	LITHIUM	VPA	TCAs
Transcription Factor CREB	↓	↓	↑		↑
Neurotrophic Factor BDNF	↓	↓	↑		↑
Neuroprotective Factor BCL-2 (Anti-Apoptotic)			↑↑	↑	
Neurite Sprouting (in vitro)		↓	↑		
Neurogenesis (in vivo)	↓	↓	↑		↑
Neuronal Viability (NAA by MRS in humans)			↑		
Increased Grey Matter (in humans)			↑		

Based on studies of Smith et al., 1995; Duman, 1998; Chen and Chuang, 1999; Gould and Tanapat, 1999; Moore et al., 2000a,b; Chuang et al., 2002

Lithium as a Neuroprotectant

Significant effects are not unique to the antidepressant substances because some mood stabilizers also appear to have neurotrophic and neuroprotective properties. Chuang and his collaborators reported neuroprotective effects of lithium in a variety of cell culture systems (Nonaka, Katsube, & Chuang, 1998) and then went on to demonstrate that this occurred *in vivo* as well. Chronic treatment with lithium in rats subjected to ligation of the middle cerebral artery (Nonaka & Chuang, 1998) reduced the size of ischemic infarct by approximately 50 percent. In addition, they observed marked neuroprotective effects of lithium in an animal model of Huntington's disease involving the intrastriatal administration of the neurotoxic compound quinolinic acid (Wei et al., 2001). For example, lithium increases the expression of BDNF and Bcl2 mRNA (two neuroprotective factors), whereas it decreases the mRNA levels of Bax and p53 (two proteins that promote cell death or apoptosis) (Chen & Chuang, 1999; Chen et al., 1999). Lithium increases neurite sprouting in culture and in humans also increases a marker of neural integrity in levels of N-acetyl aspartate (NAA) measured by MRS (Moore et al., 2000b). Taken together, these converging *in vitro* and *in vivo* data in animals and humans suggest the possibility that lithium's neuroprotective properties could be important to its therapeutic effects, although this remains to be more directly demonstrated.

Potential Liabilities of Lithium Discontinuation

These preclinical data are of great interest in relation to the clinical findings that lithium not only helps to bring the markedly elevated suicide rate in the unipolar and bipolar affective disorders back toward normal (Baldessarini, Tondo, & Hennen, 1999; Tondo et al., 1998), but also normalizes the mortality rate from associated medical conditions in patients with primary affective disorders (Ahrens et al., 1995; Coppen et al., 1991). These data raise the possibility that the antistroke and neuroprotective effects of lithium observed in animal models could play a role in the normalization of medical mortality in patients who remain on long-term lithium prophylaxis. These observations provide other secondary reasons for continuing lithium pharmacotherapy, even in the absence of a complete clinical response. New meta-analytic data from Baldessarini, Tondo, and Hennen (1999) have indicated that there is a twenty-fold increased risk of suicide in those individuals who discontinue lithium in the first year, compared with those who remain on lithium treatment.

Thus, there would appear to be a variety of potential liabilities of lithium discontinuation, including: (1) increasing the likelihood of a new episode of mania and depression and its associated morbidity; (2) provoking a serious episode requiring rehospitalization; (3) destabilizing the illness for the long term; and (4) contributing to the lethality of the illness from death by suicide. As to point 3, there are increasing data suggesting that a small percentage of patients who are doing well on lithium, but decide to discontinue their medicine and experience a relapse, will not have as robust a response when they resume treatment as they previously had (Post, Ketter, Speer, Leverich, & Weiss, 2000; Post, Leverich, Altshuler, & Mikalauskas, 1992; Post, Leverich, Pazzaglia, Mikalauskas, & Denicoff, 1993). Even in the study of Tondo et al. (1997), reporting no difference in episodes on lithium prior to and after the lithium discontinuation, a significantly higher dose of neuroleptics was required after the period off lithium.

Table 20.10. Prevalence of Lithium Discontinuation-Induced Refractoriness

Study	Length of Lithium Trial (years)	Induced Refractoriness		Patients	Notes
Post et al., 1992, 1993	6–15	9/66	13.6%	All Refractory	Depression or mania
Bauer, 1994	12	1/1	–	–	Single case
Koukopoulos et al., 1995	8.8	13/145	9%	All	Depression or mania
Maj et al., 1995	5.9	10/54	18.5%	Responders	Depression or mania; D/C refractory patients had longer lithium trials
Berghofer and Muller-Oerlinghausen, 1996	5	1/24	4.2%	All	2 initial nonresponders responded in second trial
		1/10	10.0%	Responders	Depression or mania
Tondo et al., 1997	4.6	16/86	18.6%	All	11 initial nonresponders responded in second trial; depression or mania
Coryell et al., 1998	?	1/28	3.6%	Responders	Mania
Overall incidence		39/321	12.1%	All Patients	
		12/92	13.0%	Responders Only	

In our studies and those of a variety of others, 5–15 percent of patients appeared to experience lithium discontinuation-induced refractoriness, in which even the reinstitution of lithium at higher doses than previously needed was without adequate effect (Table 20.10). Coryell et al (1998) reported little evidence for this phenomenon in their report. However, because their study: (a) was under-powered to observe this effect on a statistically reliable basis; (b) used episode criteria that were not optimal; and (c) chose subjects who were not necessarily well-established, long-term lithium responders, one wonders about the strength of the conclusions that can be drawn from that study. Moreover, (d) one of the patients reported in their study failed to re-respond, yielding a refractoriness rate of 3.6 percent even in this negative study (see Table 20.10).

Such a phenomenon of lithium discontinuation-induced refractoriness, when it does occur in an individual, can be particularly devastating, as it was for the patient illustrated in our report (Post, Leverich, Altshuler, & Mikalauskas, 1992). This patient had been completely asymptomatic for eight years during lithium monotherapy treatment, after it was initiated for a series of incapacitating depressions of 2–3 months duration as well as interposed hypomanic episodes. Ten years after restarting her lithium and subsequently adding or substituting a vast array of other treatments, she still continues to be highly symptomatic from her bipolar II illness.

Thus, in addition to the four clinical reasons enumerated above, there is a potential fifth reason for not stopping lithium prophylaxis – to the extent that lithium's neuroprotective effects are related to its mechanisms of action in the affective disorders, one could be losing such a potential long-term protective effect. Could lithium

be preventing not only episodes, but also the neural and glial loss associated with the illness, as described below? Even if lithium's neuroprotective effects were mediated separately from its therapeutic actions in bipolar affective disorders, such discontinuation of lithium might also put the patient at greater medical risk from stroke (separate from the risk of relapse, rehospitalization, refractoriness, or suicide).

Affective Illness and Brain Structure

Alterations in neurochemical content (Knable, Torrey, Webster, & Bartko, 2001; Xing et al., 2002) and in the structure of the brain (Ketter, George, Kimbrell, Benson, & Post, 1997; Soares & Mann, 1997) are increasingly being documented in the affective disorders, and therefore the potential effects of antidepressants and mood stabilizers on neurotrophic factors, neurogenesis, glial survival, and neuronal structure take on added interest. A series of studies have suggested deficient size, area, and number of glia or neurons in areas of the brain including ventral (Drevets, Ongur, & Price, 1998) and dorsal aspects of the anterior cingulate gyrus (Rajkowska et al., 1999) and mixed reports on the size of the hippocampus. Two studies (but not Pearlson et al., 1997) report increased size of the amygdala (Altshuler et al., 2000; Strakowski et al., 1999), one in proportion to the number of hospitalizations for mania (Altshuler et al., 2000).

Thus, we return to the preclinical data reviewed above indicating that experiential effects on gene expression could induce long-lasting changes in behavioral and neurochemical set points, as well as in synaptic and neuroanatomical structure and altered numbers of neuronal and glial cells. The impact of life events on brain biochemistry and microstructure could likewise be of either pathological or adaptive importance for the clinical course of the affective disorders as well. The plasticity of the brain is extraordinary and ongoing throughout one's life. With better understanding of not only the genetic underpinning of vulnerability to affective disorders, but also their interaction with crucial life events and recurrent episodes of illness, we should ultimately be able to design more rational approaches to psychological and pharmacological interventions at the appropriate opportunities.

The Possibility of Primary Prevention

As clinical and genetic markers of high vulnerability become better recognized, perhaps a role for primary prevention in those at highest risk (even before full expression of the illness) should begin to be considered. To the extent that early intervention (such as at first symptoms yielding dysfunction) helps prevent the development of more full-blown recurrent affective disorders and their progression toward treatment-refractoriness even in only a subgroup of patients, an important impact on many lives would be achieved.

Although most of the links between preclinical models and pathophysiological mechanisms in the affective disorders discussed here remain at the level of hypothesis generation and require more direct examination and testing, their ability to engender appropriate clinical questions and conceptualize the longer time domains of vulnerability (including over the entire lifetime of an individual) gives them value, even beyond their direct predictive validity. We hope that this speculative discussion will foster a wide range of questions and concepts that ultimately will lead to earlier, more focused, and rational interventions in the recurrent affective disorders. Perhaps with such early

intervention, the magnitude of the problem affective disorders pose for individuals and society – at both the level of immense suffering and in the billions of dollars they cost each year – can be very substantially lessened.

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Input-specific LTP and depotentiation in the basolateral amygdala

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The amygdala plays a central role in emotional memory. The cellular mechanisms by which the amygdala participates in emotional learning are believed to be changes in efficacy of synaptic transmission, similar to long-term potentiation (LTP) and long-term depression (LTD). Although different forms of LTP have been shown in the amygdala, many of their features are still unknown. Here, we use both field potential and intracellular recordings in rat amygdala slices, and show that LTP in the basolateral nucleus, induced by high-frequency stimulation (HFS) of the external capsule is input-specific, can be reversed by low-frequency stimulation (LFS), and can be reinstated by HFS. These synapse-specific, reversible changes in synaptic strength in the basolateral nucleus of the amygdala may be important to amygdala's role in emotional memory. *NeuroReport* 12:635–640 © 2001 Lippincott Williams & Wilkins.

Key words: Amygdala; Bidirectional plasticity; Depotentiation; Long-term depression; Long-term potentiation

INTRODUCTION

The amygdala is activated during emotional experiences via cortical and thalamic afferents to the basolateral amygdaloid compex, which includes the lateral (LA), basolateral (BLA) and basomedial (BMA) amygdaloid nuclei [1,2]. These nuclei send their output back to cortical and other limbic areas [1,3], modulating processing/interpretation and mnemonic consolidation of emotional events [4]. The LA and BLA also project to the central amygdaloid nucleus [5], which has outputs to the hypothalamus and to many areas of the brain stem [6], thus orchestrating the expression of the emotional, behavioral response [7]. Thus, a long-lasting enhancement or reduction in the responsiveness of the amygdala to cortical or thalamic inputs, may not only affect intra-amygdala processing of the incoming information, but could also have an impact on the output routes from the amygdala, affecting cortical processing and consolidation of the emotional experience via amygdalocortical pathways, as well as the input threshold for triggering an emotional response via the LA/BLA to central amygdala pathway.

Enduring changes in synaptic efficacies within the amygdala, similar to LTP and LTD, may be produced after strong and/or repetitive activation of the amygdala during stressful emotional experiences. Such long-term synaptic changes could be involved in the development and manifestation of affective disorders in humans. This view is consistent with the abnormal amygdala responsiveness to emotional stimuli in certain types of affective patients [8,9]. This view has also received support with the demonstration of LTP in the amygdala, developing in parallel with fear conditioning [10], which is a simple model for associative emotional learning, relevant to disorders of fear and anxiety. Therefore, knowledge of the characteristics and mechanisms of synaptic plasticity in the amygdala may be important to better understand the biological mechanisms underlying affective disorders.

The capacity of amygdala synapses to undergo LTP has been shown *in vitro* and *in vivo*, as well as behavioraly in association with emotional learning [11]. However, many of the properties of the different forms of amygdala LTP are still unknown. A characteristic feature of LTP, and one that qualifies LTP as a learning mechanism, is its input specificity; that is, the post-synaptic neurons display increased responsiveness to the tetanized input, but not to other converging inputs. Another feature that is shared by LTP and learning is that, for the most part, both are reversible. In the present study, we tested the input specificity and reversibility of LTP induced in the BLA by high-frequency stimulation of the external capsule (EC), the pathway that carries cortical afferents to the amygdala.

MATERIALS AND METHODS

Male, Sprague–Dawley rats, 25–35 days old were lightly anesthetized with halothane and then decapitated. Slices from the amygdala were prepared as described previously [12]. Briefly, the brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 124 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 11 glucose. A block of the brain

was prepared and glued on a Vibroslicer. Coronal slices, 500 μm thick, were cut from the amygdala, and immediately transferred to an interface chamber maintained at 33°C. Slices were perfused with ACSF at 0.5 ml/min.

Experiments were initiated about 2 h after the slices were placed in the chamber. Recordings were obtained in the BLA, while stimulation was applied to the EC and BMA (Fig. 1a). Bipolar stimulating electrodes were constructed from stainless steel wire, 50 μm diameter. Field potentials were recorded with glass pipettes filled with 2 N NaCl (2–5 MΩ), and were filtered at 1 kHz low-pass. Intracellular recordings were obtained with glass pipettes (90–120 MΩ resistance) filled with 3 M KCl. The Axoclamp 2B amplifier was used in the bridge mode. Intracellular and field signals were digitized on-line at 4 kHz.

Drugs used were 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a kainate/AMPA receptor antagonist (Research Biochemicals Int.), and D-2-amino-5-phosphonovalerate (APV), an NMDA receptor antagonist (Research Biochemicals Int.). To prepare stock solutions, APV was dissolved in dH_2O, and CNQX in DMSO (final concentration of DMSO in the slice medium was 0.01 %, v/v).

RESULTS

Field potentials: Stimulation of the external capsule (EC) evoked a fast, negative field potential in the BLA (N1, Fig. 1b, Fig. 2a) with peak latency 9.6 ± 1.3 ms ($n = 10$, mean ± s.e.). This component of the field response was probably monosynaptic, considering its relatively fast time course, and ability to follow at high frequency EC stimulation. A second, low-amplitude slow component (N2, Fig. 2a) was also present in most of the slices. In simultaneous field potential and intracellular recordings, with inter-electrode distance of < 100 μm, field potentials had a time-course similar to, although somewhat faster than the corresponding postsynaptic potentials ($n = 4$, Fig. 1b). Thus, for the most part, these field potentials were generated by extracellular sinks of synaptically evoked currents.

To obtain a control pathway for our LTP experiments we stimulated in the BMA. Single pulses to the BMA also evoked a fast, negative field potential in the BLA, which was often followed by a low-amplitude, slow component. To test whether EC stimulation and BMA stimulation activated two independent pathways we delivered paired-pulses to the two stimulation sites (one pulse to EC, the other to BMA) and compared the results with those of paired-pulse stimulation to EC (both pulses to EC), or BMA (both pulses to BMA) (Fig. 1c). The rationale of this test is that when two pulses are applied successively at a short interstimulus interval, the response to the second pulse is larger than the response to the first pulse (paired-pulse facilitation, PPF), in most synapses. This is because some free Ca^{2+} remains in the presynaptic sites for a short period of time after the first pulse, and thus more Ca^{2+} is available at these release sites when the second pulse arrives [13]. Thus, if PPF occurs when paired pulses are applied to the EC or to the BMA, but not when one pulse is delivered to EC and the second pulse to BMA (and the reverse), then it can be concluded that the two pathways do not share presynaptic sites. Paired-pulse stimulation of the EC or the BMA, at an interstimulus interval of 60 ms, produced PPF (Fig. 1c, middle traces). In contrast, when one pulse was applied to the EC and the other to BMA, no PPF was present (Fig. 1c, bottom traces). These results suggest that EC stimulation and BMA stimulation activate different afferents to BLA.

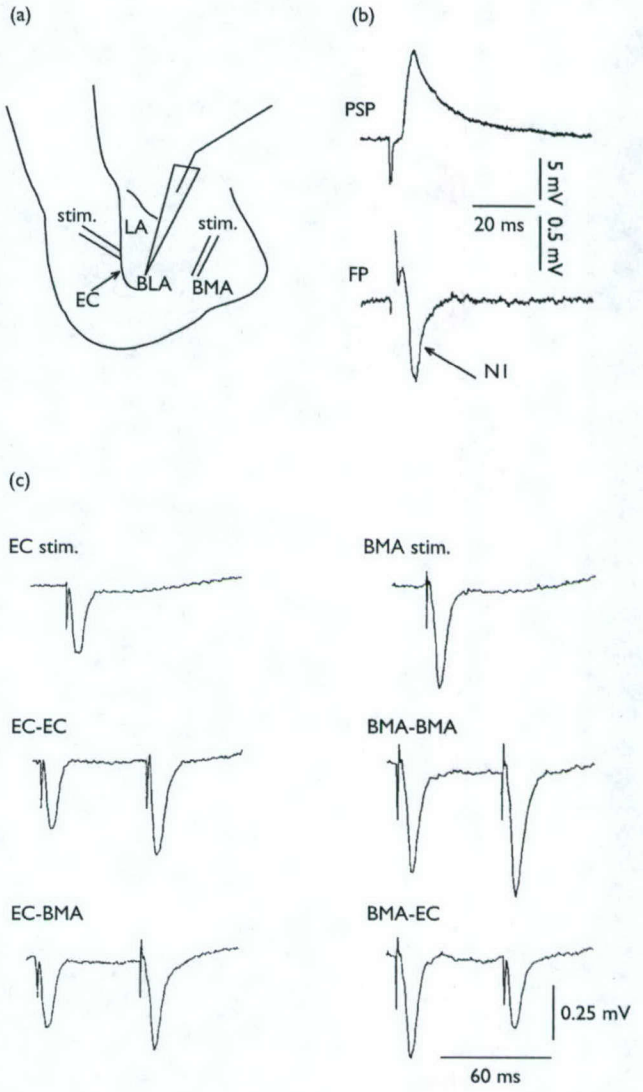

Fig. 1. (**a**) Schematic representation of the amygdala slice showing the electrode arrangement. Recordings were obtained in the basolateral amygdala (BLA), while stimulation was applied to the external capsule (EC) and basomedial amygdala (BMA). LA, lateral amygdala. (**b**) EC-evoked postsynaptic potentials (PSP) and field potentials (FP) had similar time courses. An example is shown where intracellular and extracellular recordings were obtained simultaneously. (**c**) EC stimulation and BMA stimulation activate independent pathways. Traces are field potentials recorded in the BLA in response to EC or BMA stimulation. Top traces are responses to single-pulse stimulation of the EC (left) or BMA (right). Middle traces show responses to paired-pulse stimulation of the EC (left) or BMA (right) at an interstimulus interval of 60 ms. Note the paired-pulse facilitation (PPF) in both pathways. Bottom traces show paired-pulses, when the first pulse is applied to EC and the second to BMA (left), and when the first pulse is applied to BMA and the second to EC (right). No PPF is present, and thus no evidence of interaction between the two pathways. Each trace in (b) and (c) is an average of five sweeps.

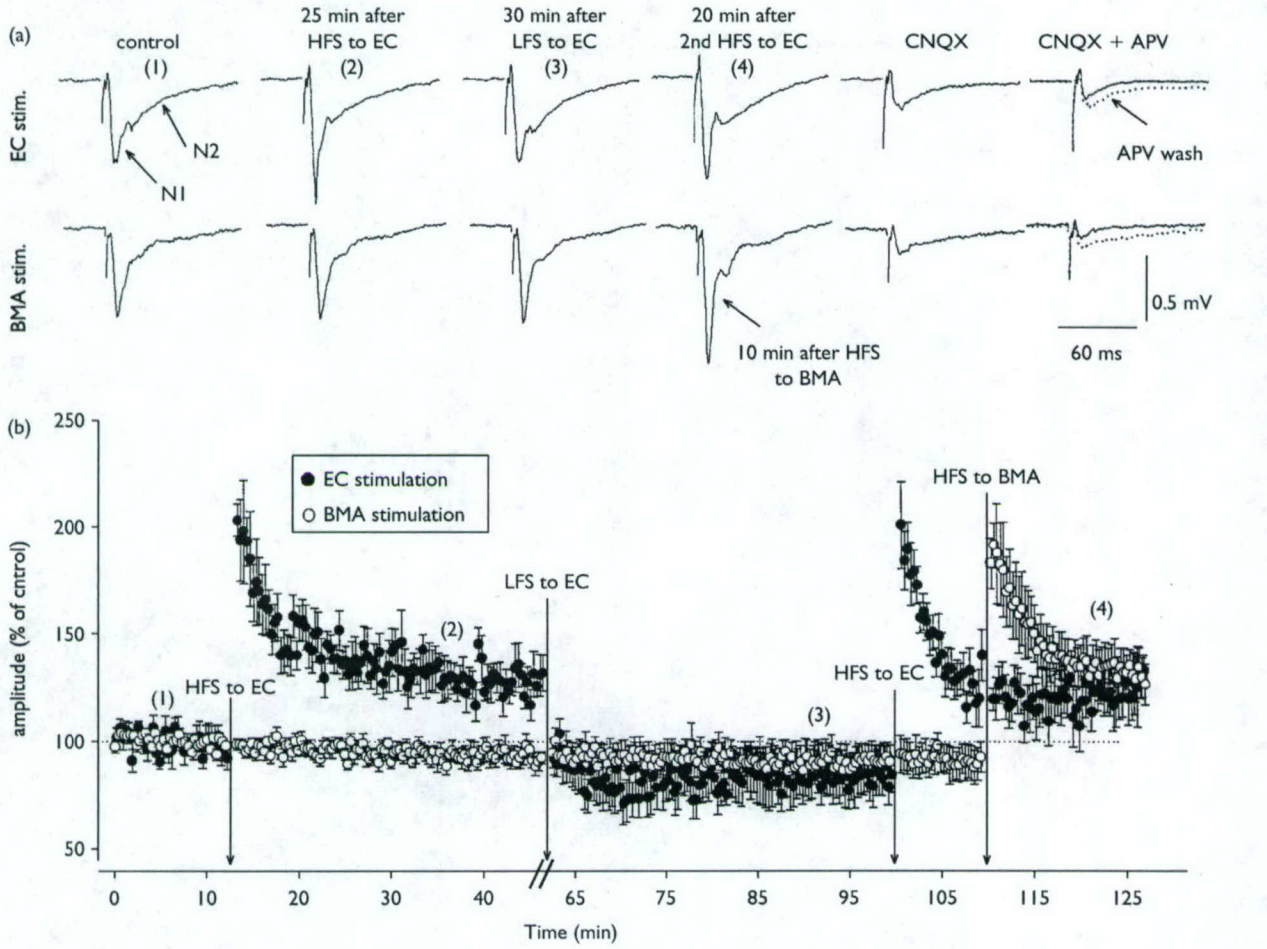

Fig. 2. Input-specific LTP and depotentiation of EC-evoked field potentials in the BLA. (**a**) Example of field potentials evoked by EC stimulation (experimental pathway, top series) and stimulation in the BMA (control pathway, lower series). (**b**) Time course of changes in the amplitude of these field potentials from eight slices (data in the plot are mean ± s.e.). Field potentials consisted of a fast component (N1) and, in most slices, a second slow component (N2). In (b) the amplitude of the fast component is plotted. HFS (two trains at 100 Hz, 1 s duration each, 20 s inter-train interval) to EC induced LTP. LFS (1 Hz, 15 min) depotentiated the responses to an amplitude below the control. The amplitude of the field potentials during 1 Hz stimulation is not shown. LTP was reinstated after a second HFS to EC. The field potentials evoked by BMA stimulation did not change significantly, but were able to potentiate after HFS to the BMA. The numbers 1,2,3 and 4 above the field potentials, and corresponding numbers on the plot indicate the approximate time points at which field potentials were averaged and depicted in (a). Each trace in (a) is an average of five sweeps. Bath application of CNQX (10 μM) nearly blocked both the EC- and BMA-evoked field potentials (a). The remaining responses were reversibly reduced by APV (50 μM).

the maximum. Thus the amplitude of N1 during baseline recordings ranged from 0.2 to 0.7 mV, with stimulus intensities 100–300 μA (150 μs duration). Using similar intensities to stimulate in BMA, the evoked field potentials had a magnitude comparable to those evoked by EC stimulation. These are very small field potentials compared to those evoked in other brain areas such as the hippocampus, neocortex or olfactory bulb; the reason is probably that the amygdala does not have the type of synaptic organization that favors generation of strong electrical dipoles in the extracellular space.

Test stimulus pulses were applied alternately to EC and BMA, with an interval of 10 s between an EC pulse and a BMA pulse. Baseline recordings were obtained for 10–15 min, which was sufficient to confirm stability of the responses. In a previous study, we found that 100 Hz stimulation of the EC, for 1 s, produces short-term potentiation of postsynaptic responses in the BLA, which did not last more than 10 min [12]. Therefore, in the present study we attempted to induce LTP by delivering two trains of pulses at 100 Hz, 1 s duration each, separated by 20 s (high-frequency stimulation, HFS). N1 was dramatically enhanced immediately after HFS (Fig. 2b), but this enhancement subsided within the next 5–7 min. This type of large, but rapidly declining, potentiation is similar to the short-term potentiation observed after a single high-frequency train to the EC [12]. The response continued to decline gradually, and reached stable potentiated levels at about 20 min post-tetanus (133 ± 7% of the control amplitude, mean ± s.e., $n = 10$). N1 remained potentiated throughout the 35 min of post-tetanus recordings. In two of the 10 slices we recorded for 1 h after HFS; N1 remained potentiated during this time. Changes in N2 were variable and were not studied systematically.

At 35 min after HFS, repetitive stimulation was applied at 1 Hz for 15 min (low-frequency stimulation, LFS). This stimulation pattern produces synaptic depression or depotentiation in other brain areas [14,15], while it causes either synaptic facilitation or depression in the amygdala, depending on the history of synaptic activity prior to LFS[12]. After LFS, N1 was depotentiated to a magnitude slightly below control (83 ± 9% of the control amplitude at 30 min post-LFS, $n = 10$). A second HFS repotentiated N1 (Fig. 2). The field potential evoked by BMA stimulation remained stable throughout, except for a gradual, modest (< 20%) depression in some slices. Thus, both LTP and depression (depotentiation) of the EC-evoked field potential were specific to the EC input. To confirm that the BMA-evoked field potential was a healthy response capable of expressing plasticity we also applied HFS to BMA. The response increased after HFS (Fig. 2) and remained at potentiated levels throughout the recording period (maximum 35 min). These results were observed in all of 10 slices. Figure 2b shows group data from eight slices because the time periods of recording were different in two slices.

At the end of three of the experiments described above we applied CNQX to the bath (10 μM). Both EC- and BMA-evoked field potentials were nearly blocked by CNQX (Fig. 2a). A small response that remained in CNQX was reversibly reduced by APV (50 μM). Thus, the field potentials were mediated for the most part by kainate/AMPA receptors, but they also included some NMDA receptor-mediated activity.

Intracellular recordings: Postsynaptic potentials (PSPs) evoked by EC or BMA stimulation were recorded from 13 neurons in the BLA. Stimulus intensities (80–200 μA) were adjusted to evoke PSPs with an amplitude about 70% of the maximum subthreshold amplitude. The peak latency of the EC-evoked PSPs was 12.6 ± 3.3 ms ($n = 8$, mean ± s.e.). Data were analyzed in eight of the 13 neurons. In two neurons, the response evoked with the lowest (threshold) EC stimulation intensity was a PSP that appeared to be polysynaptic; its onset latency was variable, particularly after HFS. In another three cells, spontaneous PSPs were very large and frequent, often coinciding with the evoked PSPs. In these neurons it was not possible to obtain reliable slope measurements of the PSPs for determining the occurrence and magnitude of LTP (see below).

The firing pattern of the cells in response to intracellular depolarizing pulses was examined both at the beginning and the end of each experiment. All cells reported here were non-bursting, with accommodating spikes, suggesting that they were probably pyramidal cells [16].

After HFS (two trains, 100 Hz, 1 s duration, 20 s inter-train interval) to the EC, the initial slope of the EC-evoked PSPs increased immediately (Fig. 3b). This enhancement subsided within the next 5–10 min, and reached stable levels at about 20 min after HFS (143 ± 11% of control, $n = 8$). Most cells frequently generated spikes over the PSP, after HFS, but this did not interfere with measurements of the initial slope (see inset in Fig. 3a). The onset latency was unaltered after HFS, suggesting that at least the early part of the PSPs was a monosynaptic excitatory response. Resting membrane potential and input resistance were monitored throughout the experiments, and they remained unchanged.

After LFS (1 Hz, 15 min) to the EC, the PSP slope returned to unpotentiated levels (95 ± 13% of control at 20 min post-LFS, mean ± s.e., $n = 8$). A second HFS to the EC repotentiated the PSPs (Fig. 3). The BMA-evoked PSPs did not change significantly when the EC-evoked PSPs increased or decreased, indicating that LTP and depotentiation were specific to the EC input. However, as in the BMA-evoked field potentials, a gradual but small depression of the BMA-evoked PSP occurred in some slices. After HFS to BMA, the BMA-evoked PSPs also increased (Fig. 3).

DISCUSSION

Previous studies have provided convincing evidence that LTP is the mechanism by which amygdala mediates emotional learning and memory [11]. Both fear conditioning and electrical HFS enhance, in the same manner, auditory stimulus-evoked field potentials in the amygdala [10]. Furthermore, behavioral fear conditioning is accompanied by LTP of AMPA receptor-mediated synaptic currents [17]. *In vitro* studies have revealed some of the mechanisms of LTP in the amygdala. HFS stimulation-induced LTP in the EC to LA synaptic transmission is, at least in part, dependent on NMDA receptors [18]. In contrast, the enduring synaptic facilitation in the EC to BLA pathway, induced by LFS is independent of NMDA receptors [12]. LTP induced by pairing thalamic presynaptic activity with postsynaptic depolarization in the LA depends on L-type Ca^{2+} channels [19], and LTP of inhibitory neuron responses to EC stimulation is mediated by AMPA receptors [20]. Evidently, there is more than one form of LTP in the amygdala. The form of amygdalar LTP may depend on which afferents are activated, which nucleus or neuron type is studied, and what pattern of stimulation is used to induce LTP. As a first step in studying HFS-induced LTP of signal transmission from the EC to BLA, we examined some of the properties of this LTP. We previously observed that a single 100 Hz train to the EC rarely induces LTP in the BLA [12]. However, the two successive trains used in the present study consistently induced LTP. Similar protocols of HFS induce LTP in the EC to LA pathway [18], but not in the thalamic input to LA [19]. In the present study, pharmacological reduction of inhibitory transmission was not necessary to induce LTP. The mechanisms underlying the induction and expression of this form of LTP remain to be determined.

The HFS-induced LTP in the EC to BLA pathway reported here was input specific. This may suggest that brief, strong BLA activation by an emotional stimulus does not cause a long-lasting enhancement of BLA responsiveness to any input, but only to the particular input that induced the increased responsiveness. In the amygdala, input specificity has also been seen in LTP induced by pairing thalamic input with post-synaptic depolarization of LA neurons in the presence of bicuculline [19], as well as in the enhancement of AMPA receptor currents in the LA after fear conditioning [17]. The mechanisms of LTP input specificity in the different amygdala nuclei are not yet known.

The ability to actively bring potentiated synapses back to upotentiated levels of synaptic strength, by applying LFS,

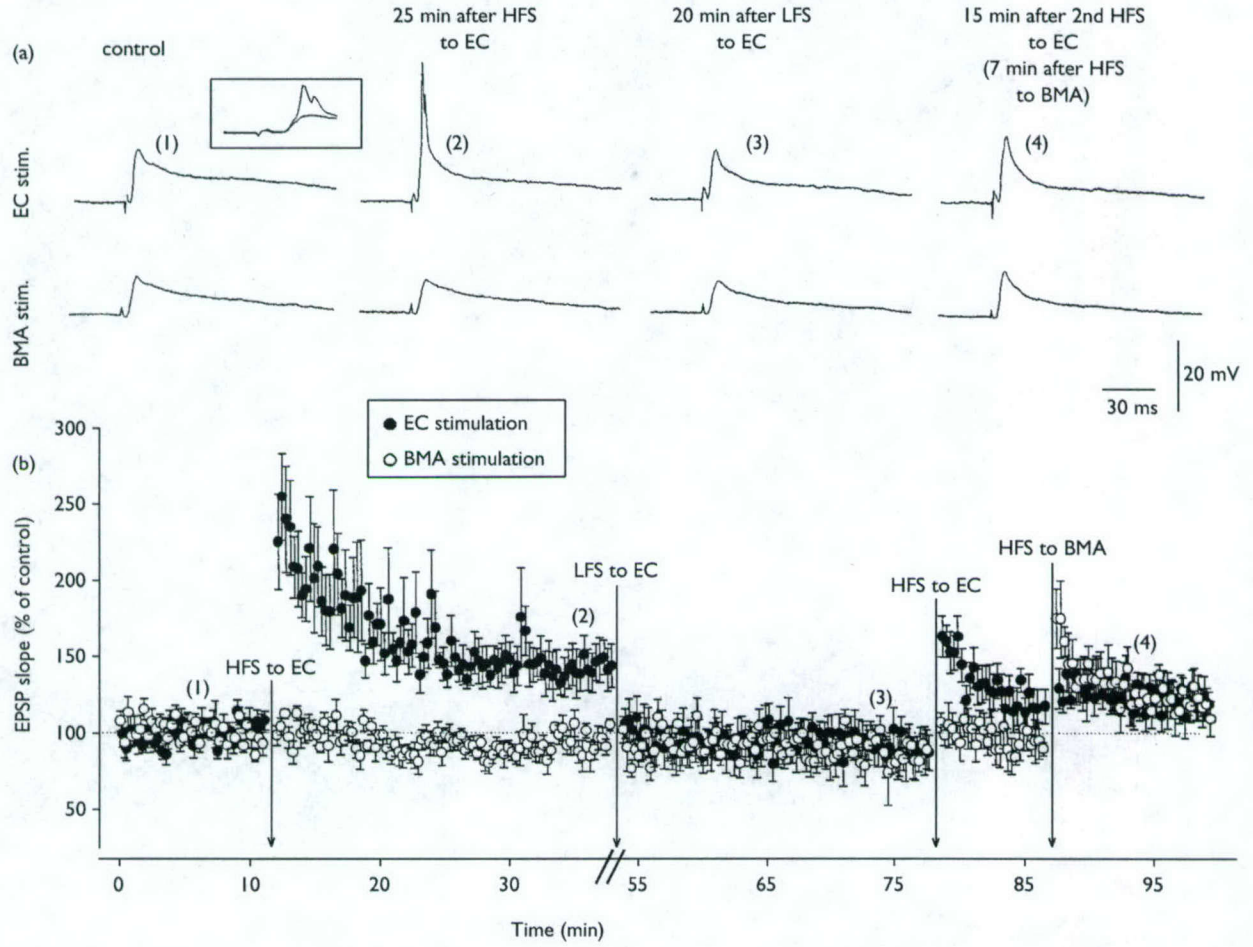

Fig. 3. Input-specific LTP and depotentiation of EC-evoked EPSPs in intracellular recordings from the BLA. (**a**) Example of PSPs evoked by EC or BMA stimulation. (**b**) Time course of changes in the initial slope of PSPs from eight slices (mean ± s.e.). HFS (two trains at 100 Hz, 1 s duration each, 20 s apart) to EC induced LTP. LFS (1 Hz, 15 min) to EC depotentiated the PSPs. LTP was reinstated after a second HFS. The PSPs evoked by BMA stimulation did not change significantly, but were able to potentiate after HFS to the BMA. The numbers 1,2,3 and 4 above the traces, and corresponding numbers on the plot indicate the approximate time points at which PSPs were averaged and depicted in (a). Each trace in (a) is an average of five sweeps. The average EC-evoked PSP shown in (2) contains spikes with shifting latencies. The inset shows the same traces as in (1) and (2), but at a faster sweep rate, to provide a better visualization of the change in slope.

has been shown in the hippocampus and visual cortex [14,15]. Depotentiation has not been shown previously in the amygdala. In naive amygdala slices, LFS induces NMDA- and metabotropic receptor-dependent LTD in the LA to BLA pathway [21], but NMDA receptor-independent LTP in the EC to BLA pathway [12]. In the same EC to BLA pathway, LFS induces LTD if applied after a single 100 Hz that does not produce long-lasting effects [12]. In the present study, we find that when LFS is applied after LTP induction it produces depotentiation. Some of the mechanisms by which LFS produces different effects in the BLA depending on the history of previous synaptic activity have been revealed [12] and are currently under investigation.

Depotentiation in the amygdala may have clinical relevance. In some types of clinical depression, patients exhibit hypermetabolism in the amygdala [22,23], or exaggerated amygdalar responses to emotional stimuli [8]. It is possible that hyperresponsiveness of the amygdala in such patients could be reduced if the amygdala were activated repetitively at low frequency. Repetitive transcranial magnetic stimulation [24] at low frequency (1 Hz) has been effective in reducing amygdala hyperactivity and improving mood in certain patients [25].

CONCLUSION

We showed that LTP in the basolateral amygdala, induced by two, brief, high-frequency trains of pulses to the external capsule is input specific. This suggests that, at least when the amygdala receives strong stimulation for only a brief period of time, there is no generalized increased amygdala excitability; instead, increased responsiveness is limited to the input that induced it. We also showed that it is possible to actively depotentiate synapses in the basolateral amygdala by applying LFS to the potentiated pathway, and to reinstate LTP by subsequent

HFS. Thus, as in the hippocampus, the strength of synaptic transmission in the basolateral amygdaloid nucleus can reversibly increase or decrease depending on the activity pattern of cortical afferents.

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Kainate receptor mediated heterosynaptic facilitation in the amygdala

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Prolonged low-frequency stimulation of excitatory afferents to basolateral amygdala neurons results in enduring enhancement of excitatory synaptic responses. The induction of this form of synaptic plasticity is eliminated by selective antagonists of GluR5 kainate receptors and can be mimicked by the GluR5 agonist ATPA. Kainate receptor-mediated synaptic facilitation generalizes to include inactive afferent synapses on the target neurons, and therefore contrasts with other types of activity-dependent enduring synaptic facilitation that are input-pathway specific. Such heterosynaptic spread of synaptic facilitation could account for adaptive and pathological expansion in the set of critical internal and external stimuli that trigger amygdala-dependent behavioral responses.

Activity-dependent changes in synaptic efficacy are believed to underlie the long-term functional modifications in brain circuits that allow behavior to be altered in response to experience. In the best studied examples of activity-dependent enduring synaptic plasticity, synaptic strengthening is produced by high-frequency stimulation, whereas weakening may occur with low-frequency stimulation, and induction of both forms of synaptic plasticity requires activation of *N*-methyl-D-aspartate (NMDA)-type ionotropic glutamate receptors[1–3]. However, in basolateral amygdala neurons, prolonged low-frequency stimulation of the external capsule (EC) produces an enduring enhancement in the amplitude of EC-evoked excitatory synaptic responses[4]. EC stimulation activates excitatory afferents from cortical structures, including the lateral entorhinal and temporal cortices, that course through the EC and synapse in the basolateral amygdala[5,6]. A key function of the amygdala is to attach emotional significance to the secondary sensory and contextual information carried by these afferents[7]. Here we show that induction of enduring synaptic enhancement in basolateral amygdala neurons by low-frequency EC stimulation requires activation of GluR5 kainate-type ionotropic glutamate receptors acting through a Ca^{2+}-dependent mechanism. There is also enhancement in the strength of excitatory synaptic responses evoked by a convergent, unstimulated input pathway from the basal amygdala (BA). Kainate receptor-mediated synaptic facilitation contrasts with other types of activity-dependent enduring synaptic facilitation (such as hippocampal long-term potentiation) in that it develops slowly and generalizes to include inactive afferent synapses on the target neuron. Such heterosynaptic facilitation provides a means by which the set of stimuli associated with a behavioral response can be broadened instead of sharpened, as occurs with conventional input-specific synaptic plasticity mechanisms.

Results

GluR5 kainate receptors mediate low-frequency stimulation-induced synaptic facilitation[author:please cut to 1 line]

Low-frequency stimulation of the EC (1 Hz, 15 min) in rat brain slices containing the amygdala elicited a slowly progressive enhancement in the amplitude of EC-evoked excitatory synaptic potentials recorded intracellularly in basolateral amygdala neurons (**Fig. 1a**). The increase in synaptic response amplitude began during the stimulus train and continued for 20 to 30 min following its termination. In a series of 6 experiments, the peak synaptic response amplitude 30 min after completion of the stimulus train was 219 ± 13% of baseline. To eliminate the possibility that the increase was exclusively due to recruitment of polysynaptic responses or to depression of synaptic inhibition, we determined the initial slope of the excitatory synaptic responses. Low-frequency stimulation was associated with an increase in the initial slope that followed a similar time course as the increase in peak response amplitude (**Fig. 2a** and **b**). There was also a small reduction in the mean latency to the onset of the synaptic response (5.1 ± 0.4 and 4.1 ± 0.4 ms; $n = 9$; $p < 0.001$). The enhancement of the synaptic response occurred in the absence of any change in the cell's resting potential or in its passive or active membrane properties. In the 6 experiments of Fig. 1a, the mean resting potential values before and after stimulation were 68 ± 1 and 67 ± 1 mV, respectively. In these experiments, the mean input resistance values determined from the voltage response to 100 pA hyperpolarizing current pulses injected through the recording electrode were, respectively, 59.7 ± 2.0 and 58.3 ± 2.1 MΩ. In some experiments, we also monitored voltage responses and action potential firing evoked by depolarizing current steps. Low-frequency stimulation-induced synaptic enhancement was not associated with any change in the amplitude of the peak or steady-state voltage response, or in the

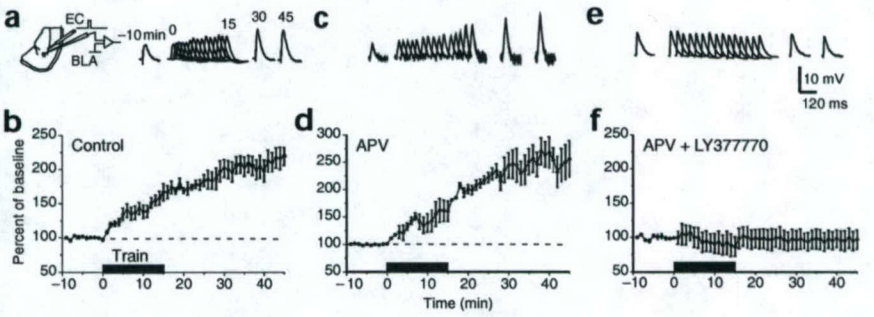


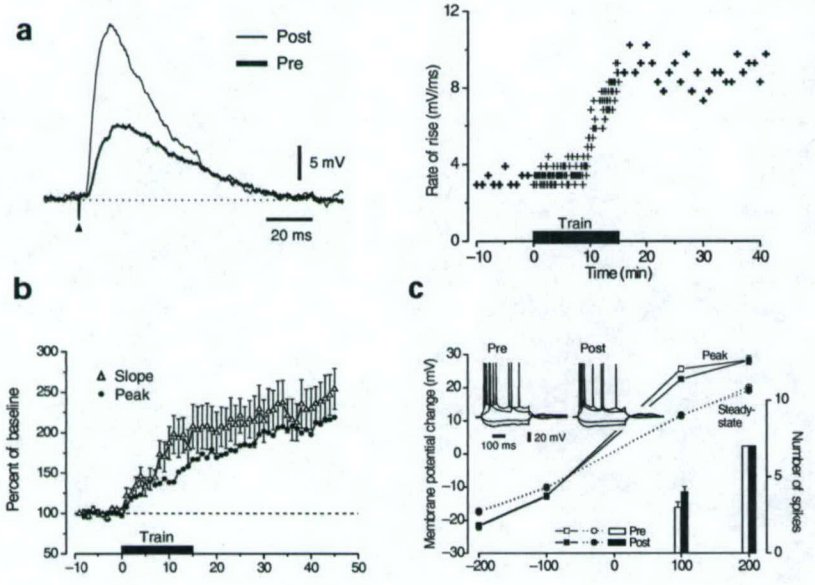
Fig. 1. Low-frequency external capsule (EC) stimulation induces long-term facilitation of the excitatory synaptic response in basolateral amygdala (BLA) neurons. **(a)** Left, stimulation and recording configuration in the amygdala slice. Right, intracellularly recorded synaptic responses at various times before, during and after train stimulation at 1 Hz for 15 min. Traces before (−10 min) and after (30 and 45 min) stimulation are the averages of 6 consecutive responses to stimulation at 0.1 Hz; traces during stimulation are the averages of 60 responses. Train stimulation causes an enhancement in the amplitude of the synaptic response without changing the latency. **(b)** Peak synaptic potential amplitude as a percent of the amplitude at −10 min. For each experiment, cumulative averages were obtained during 1-min epochs (6 or 60 successive responses). Each point represents the mean ± s.e.m. of the averaged response amplitudes from 6 experiments. Train stimulation was applied during the 15-min period indicated by the bar. **(c)** Inclusion of 100 μM APV in the perfusion solution fails to interfere with the development of low-frequency train-induced long-term facilitation in an experiment similar to that described in **(a)**. **(d)** Summary of data from 6 experiments with 100 μM APV. **(e)** Inclusion of 20 μM LY377770 in the perfusion solution (in the presence of 100 μM APV) eliminates the development of low-frequency train-induced long-term facilitation in an experiment similar to that described in **(a)**. **(f)** Summary of data from 6 experiments with 100 μM APV and 20 μM LY377770.

input–output relationship for action potential firing, indicating that the cell's membrane properties are unaltered in the voltage region relevant to excitatory synaptic responses (Figs. 2c and 7b).

To determine if induction of the synaptic enhancement is dependent upon NMDA receptors, recordings were carried out in the presence of the NMDA receptor antagonist D(−)-2-amino-5-phosphonopentanoate (APV). Perfusion of the slices with 100 μM APV did not alter the amplitude of single evoked synaptic responses and failed to affect the development of low-frequency train-induced synaptic facilitation (Fig. 1b). In 6 experiments where APV was included in the perfusion solution, the peak synaptic response amplitude 30 min after termination of the stimulus trains was 256 ± 34% of baseline, which is not significantly different from the value obtained in the absence of APV ($p > 0.05$).

We have previously reported that a component of the synaptic depolarization evoked by EC stimulation in basolateral amygdala neurons is mediated by GluR5 kainate receptors[8]. This component is resistant to NMDA receptor antagonists and to 2,3-benzodiazepine-selective AMPA receptor antagonists[9,10], but is blocked by decahydroisoquinoline GluR5-selective kainate receptor antagonists[11,12]. *In situ* hybridization histochemistry reveals dense expression of GluR5 mRNA in a patchy distribution in the amygdala and adjacent piriform cortex, with especially high levels at the site of recording in the basolateral nucleus (Fig. 3). GluR5 expression levels are higher in the amygdala than in the hippocampal formation, whereas the opposite applies for GluR6 and KA-2 mRNA, as noted by others[13]. The results from *in situ* hybridization were confirmed by reverse transcriptase PCR (RT-PCR) of GluR5 mRNA extracted from microdissections of

Fig. 2. Effects of low-frequency train stimulation on the initial slope of EC-evoked synaptic responses and on cell firing in response to injected current. **(a)** Left, comparison of the EC-evoked synaptic response before (Pre) and 30 min after (Post) the termination of EC train stimulation at 1 Hz for 15 min. Arrowhead marks the onset of the EC test stimuli. Right, maximum rate of rise (after achieving 10% of peak amplitude) of the synaptic response expressed as the averages of 6 (before and after train stimulation; thick symbols) or 60 (during train stimulation; thin symbols) successive responses. **(b)** Cumulative averages of rate of rise values during 1-min epochs (6 or 60 successive responses) expressed as a percent of the value at −10 min. White triangles represent mean ± s.e.m. of cumulative average values from eight experiments including those described in **(a)**. The peak amplitude values from **(a)** are shown for comparison by black circles. **(c)** Response of a BLA neuron to injected current (±100 pA, ±200 pA) before and 28 min after synaptic facilitation induced by EC train stimulation (1 Hz, 15 min). The mean ± s.e.m. amplitudes of the peak and steady-state (measured at end of current steps) membrane potential deflections are shown with squares and circles, respectively; mean number of spikes are shown with bars ($n = 5$). White symbols, before (Pre); black symbols, after (Post) train stimulation. There is no significant difference in the mean peak or steady-state responses, or in the number of spikes, with any of the injected current values. Inset, sample traces. Resting potential, −70 mV.



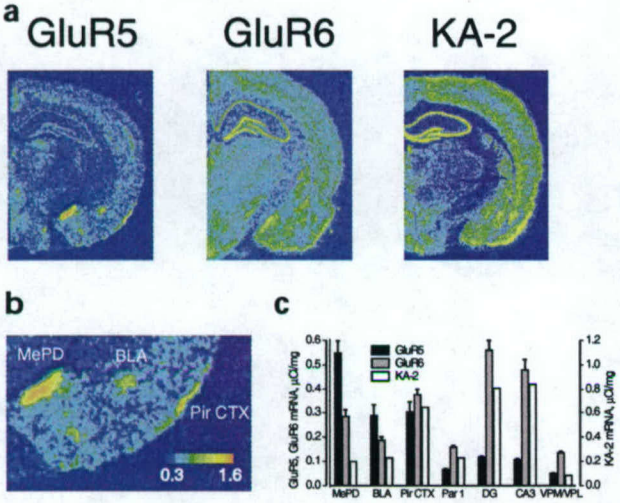


Fig. 3. GluR5, GluR6 and KA-2 kainate receptor mRNA expression by *in situ* hybridization histochemistry in rat brain sections at the level of the BLA. **(a)** Pseudocolor images showing distinct regional distributions of the subunit mRNAs. Scale to expression levels in μCi/mg appears in **(b)**. **(b)** Enlarged view of temporal lobe region showing dense expression of GluR5 mRNA in MePD and BLA amygdala nuclei and Pir CTX. **(c)** Quantification of kainate receptor subunit mRNA expression in five brain regions. GluR5 mRNA expression is highest in temporal lobe structures, including the BLA, whereas GluR6 and KA-2 mRNA is highest in the hippocampal formation. MePD, posterodorsal medial amygdala; BLA, basolateral amygdala; Pir CTX, piriform cortex; Par 1, parietal cortex; DG, dentate gyrus; CA3, hippocampal CA3 subfield; VPM/VPL, ventroposteromedial and ventroposterolateral nuclei of the thalamus. Brain regions are defined according to the atlas of Paxinos and Watson[50], with the BLA located in relationship to the EC and stria terminalis.

slices. With respect to amygdala, hippocampus contained lower GluR5 mRNA content (38.4 ± 4.7%; mean ± s.e.m.; $n = 6$).

We next investigated whether the kainate receptor-mediated component of the synaptic response could contribute to the induction of LFS-induced synaptic enhancement, using the decahydroisoquinoline-selective GluR5 kainate receptor antagonist LY377770 (ref. 14). In 5 experiments studying the EC-evoked excitatory synaptic response in the presence of APV, perfusion with GYKI 53655 at a concentration of 50 μM, which blocks AMPA but not kainate receptors[9,10], caused a progressive decline in the synaptic response to a minimum steady-state level at 20 min of 23% of the baseline response (Fig. 4). Along with the GYKI 53655, we also included the $GABA_A$ and $GABA_B$ receptor antagonists bicuculline and SCH 50911 (ref. 15) to eliminate GABA-mediated synaptic responses. Subsequent addition of 20 μM LY377770 nearly abolished the synaptic response (2% of control). Although LY377770 predominantly blocks GluR5 kainate receptors, it can also affect AMPA receptors. However, in the presence of AMPA receptor blockade with GYKI 53655, the component subtracted by LY377770 provides a representation of the GluR5 kainate receptor contribution.

Inclusion of 20 μM LY377770 in the perfusion solution eliminated the enhancement induced by low-frequency train stimulation (Fig. 1c). In these experiments, 100 μM APV was present in the perfusion solution so that the evoked synaptic response was predominantly mediated by AMPA receptors with a small component mediated by kainate receptors. In 4 experiments, the peak synaptic response amplitude 30 min after termination of the stimulus train was 97 ± 14% of the initial baseline amplitude, which is significantly different from the values obtained in the absence of LY377770 ($p < 0.01$). These observations indicate that GluR5 kainate receptors are required for the induction of low-frequency stimulation-induced synaptic facilitation in basolateral amygdala, a conclusion supported by further studies examining the effects of LY377770 on stimulation-induced facilitation of the NMDA receptor-mediated component of the synaptic response in the presence of AMPA receptor blockade (see below).

Direct activation of GluR5 kainate receptors induces synaptic facilitation [author: Please cut to 1 line]

Low micromolar concentrations of ATPA [(*R*,*S*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl] predominantly activate GluR5 kainate receptors, but not AMPA or GluR6 kainate receptors[12]. Perfusion of amygdala slices with 20 μM ATPA initially caused a small, transient depression in the amplitude of EC-evoked synaptic responses (Fig. 5a). However, within 8 to 10 min, the synaptic response amplitude recovered and continued to increase even after termination of the perfusion. In 4 experiments, the mean synaptic response amplitude reached a plateau level 218 ± 33% of the baseline value, 30 min after the onset of the ATPA perfusion. There was no decrement in the enhanced synaptic response, and the potentiated response persisted as long as the recording could be maintained (in some cases, greater than 120 min). The ATPA-potentiated synaptic depolarization was markedly prolonged and seemed to consist of multiple components, suggesting that ATPA caused a recruitment of excitatory synaptic potentials. The onset of the synaptic response followed the stimulus with high fidelity, indicating that the initial component is monosynaptic. Furthermore, following ATPA perfusion, there was a persistent increase in slope, which exceeded the increase in peak amplitude (Fig. 5b). However, as with low-frequency stimulation, there was a small decrease in mean latency from 4.5 ± 0.6 ms before stimulation to 3.7 ± 0.5 ms after ATPA perfusion ($p < 0.001$). In 4 additional experiments, inclusion of the LY377770 analog LY382884 in the perfusion solution eliminated the delayed enhancement of the synaptic response. (LY382884 was available to us in only limited quantity; it has similar affinity for GluR5 kainate receptors as LY377770, but has 15-fold reduced affinity for AMPA receptors[14,16].) However, the initial ATPA-induced depression was not blocked, and, in fact, was greater

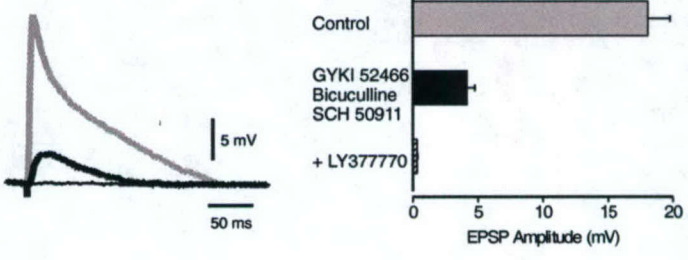


Fig. 4. GluR5 receptor-mediated component of the EC-evoked excitatory synaptic response. **(a)** Sample traces comparing control synaptic response (gray trace) with synaptic responses 10 min following inclusion in the perfusion solution of 50 μM GYKI 53655, 10 μM bicuculline and 10 μM SCH 50911 (thick black trace), and 10 min following the additional inclusion of 20 μM LY377770 (thin black trace). The control solution contained 100 μM APV. **(b)** Mean ± s.e.m. peak synaptic response amplitudes in 5 experiments similar to **(a)**.

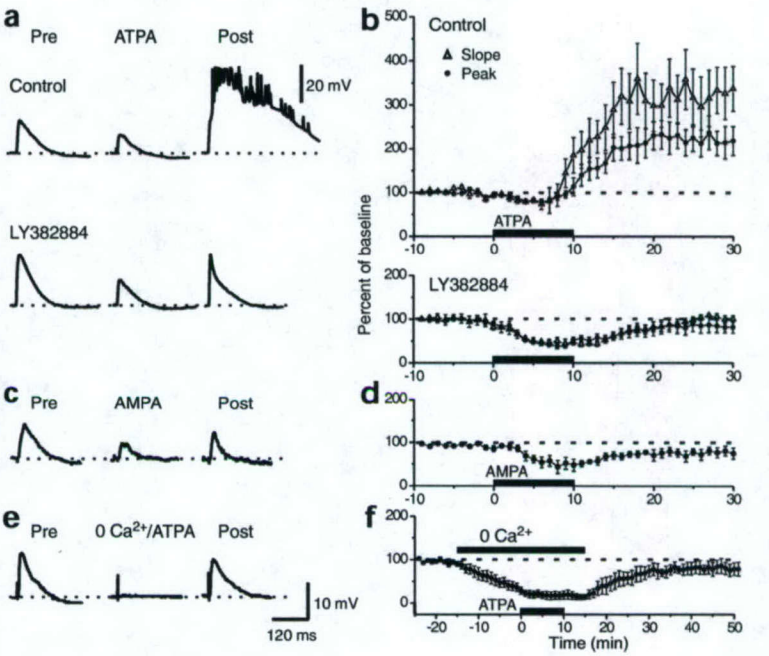


Fig. 5. ATPA but not AMPA induces persistent synaptic facilitation that is eliminated by LY382884 and perfusion with Ca^{2+}-free solution. **(a)** Sample traces showing effects of perfusion with 20 μM ATPA on synaptic responses evoked by EC stimulation in the absence (top) and presence (bottom) of 200 μM LY382884. Traces are the averages of 6 consecutive responses to stimulation at 0.1 Hz. 'Pre' traces were acquired 10 min before the start of the ATPA perfusion; middle (ATPA) traces were acquired 7 min after the start of the ATPA perfusion; 'Post' traces were acquired 15 min after the termination of the ATPA perfusion. Note the reduced scale of the Control/Post trace as indicated by the bar that applies to this trace only. There is a moderate reduction in the amplitude of the synaptic response during ATPA perfusion in the absence and presence of LY382884. **(b)** Summary of 4 experiments similar to those described in **(a)** in the absence (top) and presence (bottom) of 200 μM LY382884. Black circles indicate peak amplitude and open triangles represent the initial slope. **(c, d)** Similar experiments to those described in **(a)** and **(b)** except with 2 μM AMPA instead of ATPA. There is a reduction in the amplitude of the synaptic response during AMPA perfusion. The results of three experiments are summarized in **(d)**. **(e)** Sample traces showing effects of perfusion with 20 μM ATPA on synaptic responses evoked by EC stimulation during perfusion with solution containing no added Ca^{2+} and 1 mM EGTA. Traces are the averages of 6 consecutive responses to stimulation at 0.1 Hz. 'Pre' traces were acquired 15 min before start of Ca^{2+}-free (0 Ca^{2+}) perfusion; middle (ATPA) traces were acquired 8 min following onset of 20 μM ATPA perfusion (during 0 Ca^{2+} perfusion); 'Post' traces were acquired 35 min after termination of 0 Ca^{2+} perfusion (40 min after termination of ATPA). **(f)** Summary of 7 experiments similar to those in **(e)** **[Author: OK?]**.

in the absence of the potentiation, indicating that the depressant effect of ATPA is not mediated through GluR5 kainate receptors.

ATPA, the *tert*-butyl analog of AMPA, although relatively selective for GluR5 kainate receptors, is not entirely free of AMPA receptor agonist activity, and does bind to AMPA receptors at 10- to 100-fold higher concentrations than AMPA itself[17,18]. Therefore, to exclude the possibility that the facilitatory effects of ATPA are related to activation of AMPA receptors, we carried out a comparable series of experiments with AMPA at a concentration (2 μM) having AMPA receptor agonist activity roughly equivalent to the AMPA receptor agonist activity of 20 μM ATPA. In these experiments, AMPA produced a moderate depression of evoked synaptic responses (Fig. 5c and d). At 10-fold higher AMPA concentrations, neurons were strongly depolarized and most recordings could not be maintained, possibly due to an excitotoxic action of these high AMPA concentrations. In no case, however, did we observe a delayed facilitation of the synaptic response.

Heterosynaptic nature of LFS-induced synaptic facilitation [author: Please cut to 1 line]

The best-studied forms of activity-dependent synaptic facilitation are input-pathway specific as is required for Hebbian learning[19]. In basolateral amygdala neurons, brief high-frequency tetanic stimulation of the EC (100 Hz, 1 s) produces NMDA receptor-dependent short-term potentiation of the synaptic response that typically persists for no more than 10 min[4]; a second high frequency stimulation train applied shortly thereafter (within ~20 s) often induces sustained (long-term) synaptic potentiation[20]. Both forms of high-frequency tetanus-induced synaptic plasticity are input-pathway specific as can be demonstrated by alternately assessing the synaptic strength via two stimulating electrodes, the first in the EC and the second in the basal amygdala (BA), a site that provides a distinct monosynaptic afferent excitatory input to basolateral amygdala neurons[21,22]. The possibility of current spread between the two input pathways was eliminated by examining paired-pulse facilitation. Short-latency paired stimulation of the EC resulted in a potentiation of the response to the second stimulus; no similar enhancement was observed when the second stimulus was applied to the BA[20]. In addition, in six experiments, short-term potentiation of the EC input to basolateral amygdala neurons induced by a single high frequency tetanus was not associated with any change in responsiveness to BA stimulation (data not shown).

To determine if low-frequency stimulation-induced synaptic facilitation is also input-pathway specific, similar experiments were carried out, in which the EC was subjected to 15 min of low-frequency train stimulation. In some cases, we monitored the non-NMDA receptor-mediated component of the synaptic response (Fig. 9c). In other experiments, the NMDA receptor component of the synaptic response was isolated by inclusion of 50 μM GYKI 53655 in the perfusion solution, which provides an opportunity to eliminate the possibility that LY377770 perturbs synaptic facilitation through non-selective effects on AMPA receptors. In hippocampal CA1 neurons, activation of group I metabotropic glutamate receptors (mGluR) has been demonstrated to produce a slow enhancement of NMDA receptor responses[23]. Therefore, to eliminate the possibility that group I mGluR activation contributes to the facilitation of NMDA receptor-mediated synaptic responses, the group I mGluR selective, noncompetitive antagonist 7-(hydroxyimino)cyclopropa[b]-chromen-1a-carboxylate ethylester (CPCCOEt) was included in the perfusion solution[24]. In the presence of these pharmacological antagonists (and a compensatory increase in stimulation intensity), excitatory synaptic responses were more prolonged (227 ± 6 ms, $n = 16$ versus 99 ± 5

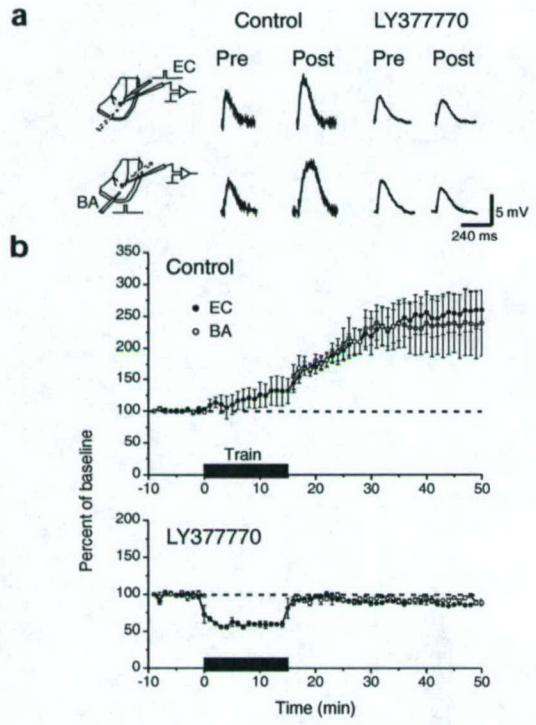


Fig. 6. Low-frequency train-induced heterosynaptic facilitation of NMDA receptor-mediated synaptic responses. **(a)** Left, sites for intracellular recording in the basolateral amygdala and dual pathway stimulation of the external capsule (EC) and basal amygdala (BA). Right, synaptic responses evoked by EC (top) and BA (bottom) stimulation before ('Pre') and 10 min after ('Post') low-frequency train stimulation (1 Hz, 15 min) of the EC in separate experiments without (control) or with 20 μM LY377770 in the perfusion solution. The perfusion solution also contained 50 μM GYKI 53655, 10 μM bicuculline, 10 μM SCH 50911 and 20 μM CPCCOEt. Traces are averages of 3 synaptic responses. **(b)** Top, mean peak synaptic response amplitude as percent of the amplitude at −10 min of EC- and BA-evoked synaptic responses from 4 experiments. Low-frequency train stimulation was applied to the EC during the 15-min period indicated by the bar; BA responses were not evoked during the train stimulation. Bottom, mean peak synaptic response amplitude as percent of the amplitude at −10 min of EC- and BA-evoked synaptic responses from 4 experiments in which 20 μM LY377770 was present in the bathing solution. There is a reduction in the EC-evoked synaptic response amplitude during train stimulation with recovery nearly to baseline upon termination of the train. Stimulation intensities were equivalent in the experiments with and without LY377770.

ms, $n = 28$) and were largely but not completely (~75%) eliminated by APV, indicating that they are predominantly mediated by NMDA receptors. The residual synaptic response was blocked by perfusion with LY377770, and is therefore due to activation of GuR5 kainate receptors. As is apparent in the experiment of Fig. 6, low-frequency train stimulation caused a slow, delayed enhancement of the predominantly NMDA receptor-mediated EC-evoked synaptic response recorded in the presence of 50 μM GYKI 53655. Thus, low-frequency train stimulation of basolateral amygdala neurons induces a comparable facilitation of the non-NMDA and NMDA components of the synaptic response, as is the case for hippocampal mossy fiber long-term potentiation[25], another type of NMDA receptor-independent form of synaptic plasticity. In addition, however, there was a gradual enhancement of the response evoked by activation of the naive BA input, which paralleled the enhancement of the EC-evoked response. In 4 such experiments, the mean homosynaptic (EC) and heterosynaptic (BA) potentiation 30 min after termination of the stimulus train was 259 ± 31% and 239 ± 51% of the initial baseline amplitude, respectively. The synaptic response enhancement in both pathways could be eliminated by inclusion of 20 μM LY377770 in the perfusion solution (Fig. 6); in 3 experiments, the mean homosynaptic and heterosynaptic potential amplitudes 30 min after termination of the stimulus train were 88 ± 4% and 88 ± 5% of the initial baseline amplitudes, respectively. There were significant differences between the magnitudes of the potentiations in the experiments with and without LY377770 ($p < 0.01$). We have demonstrated that LFS is not associated with a change in passive or active membrane properties of BLA neurons, indicating that the enhanced amplitude of the synaptic response evoked from the naive input is not due to an increase in cell input resistance resulting in a larger voltage response for the same synaptic current. However, to insure the stability of the cell excitability characteristics during the development of heterosynaptic facilitation, in most experiments we continuously monitored the passive and active cell properties by injecting hyperpolarizing and depolarizing current. Heterosynaptic facilitation was not associated with a general change in cell excitability (Fig. 7).

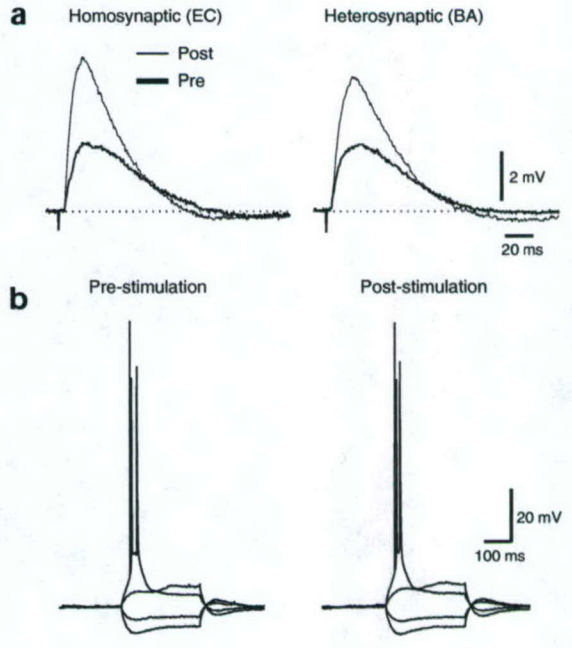


Fig. 7. Heterosynaptic facilitation is not associated with a change in membrane properties of the postsynaptic neuron. **(a)** Synaptic responses in a BLA neuron evoked by EC (homosynaptic) and BA (heterosynaptic) stimulation before ("Pre") and 28 min after the termination of low-frequency EC stimulation (1 Hz, 15 min). The peak amplitudes of the EC- and BA-evoked post-stimulation responses are 229 and 202% of the pre-stimulation values, respectively. There was post-stimulation increase in the rate of rise in both the homosynaptic (114%) and heterosynaptic (56%) responses. **(b)** Responses of the neuron to injected current (±50 pA, ±100 pA) at the times the synaptic responses in **(a)** were acquired demonstrating the lack of change in passive or active membrane properties. Resting potential, −75 mV.

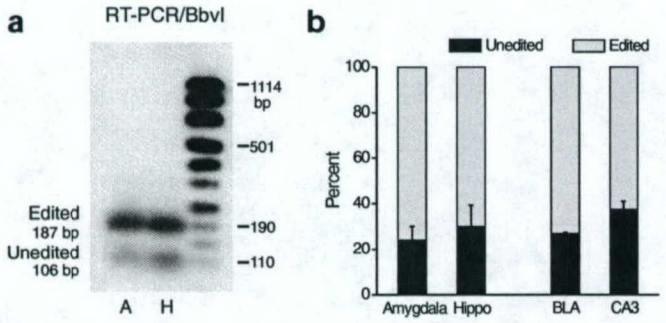


Fig. 8. Editing of GluR5 mRNA extracted from microdissections of amygdala and hippocampus. Total RNA was subjected to RT-PCR, the PCR product was digested with the endonuclease BbvI, separated on agarose gels and detected by Southern blotting. **(a)** Sample blots showing extent of editing of GluR5 mRNA in amygdala (A) and hippocampus (H) dissected from brain slices. **(b)** Summary of data from three separate experiment experiments similar to that shown in **(a)**. In addition, data are provided from three experiments where the brain slice dissections were limited to the basolateral amygdala (BLA) and hippocampal CA3 region.

BA stimulation was previously reported to evoke a smaller GluR5 kainate receptor component of the synaptic response than EC stimulation[8]. Consonant with the role of GluR5 kainate receptors in mediating heterosynaptic facilitation, low-frequency stimulation of the BA less consistently induced facilitation of BA- and EC-evoked synaptic responses (3 of 6 experiments; data not shown).

Role of Ca^{2+} in heterosynaptic facilitation

GluR5 kainate receptor pre-mRNA undergoes post-transcriptional nuclear editing, leading to substitution of an arginine (R) for a glutamine (Q) at residue 636 (Q/R site) within the second hydrophobic (MII) domain of the subunit protein[26,27]. By analogy with AMPA and kainate receptors composed of GluR2 and GluR6 subunits[28–30], GluR5 kainate receptors containing unedited subunits are expected to be more Ca^{2+} permeable than those composed entirely of edited subunits. In adult brain, the fraction of unedited GluR5 mRNA is generally greater than 30% (ref. 31), suggesting that native GluR5 kainate receptors are often Ca^{2+} permeable. To determine the extent of editing of GluR5 mRNA in the BLA, RNA was extracted from microdissections of this region and subjected to RT-PCR followed by restriction endonuclease cleavage with BbVI[31]. Twenty-seven percent of the GluR5 mRNA in dissections limited to the region of the BLA was unedited (Fig. 8). A comparable fraction was unedited in whole amygdala microdissections and also in microdissections of the CA3 region of the hippocampus, where GluR5 kainate receptors have also been implicated in synaptic transmission and plasticity[16,32,33].

A requirement for Ca^{2+} influx in GluR5 kainate receptor-mediated synaptic facilitation is suggested by experiments demonstrating that ATPA-induced enhancement of the synaptic response failed to occur when ATPA was applied during perfusion with solution lacking Ca^{2+} (Fig. 5c). To assess a role for intracellular Ca^{2+} in low-frequency stimulation-induced heterosynaptic facilitation, amygdala neurons were loaded with the Ca^{2+} chelator BAPTA by perfusion for 30 min with its cell-permeable acetoxymethyl derivative BAPTA-AM, which is activated intracellularly by nonspecific esterases[34]. BAPTA-AM treatment did not alter the input resistance (before BAPTA-AM, 60.5 ± 5.9 MΩ; after BAPTA-AM, 58.6 ± 5.3 MΩ; 8 cells] or excitability properties of basolateral amygdala neurons, although there was an acceleration in the rate of rise and decay of the synaptic response and a modest reduction in the amplitude of the evoked synaptic responses (EC, 19 ± 11%; BA, 24 ± 10%) that could be overcome by increasing the stimulation intensity (Fig. 9a). The heterosynaptic enhancement of EC- and BA-evoked synaptic responses induced by low-frequency stimulation no longer occurred after BAPTA-AM treatment (Fig. 9b and c). These results indicate that enduring synaptic enhancement in basolateral amygdala neurons requires a rise in intracellular Ca^{2+}.

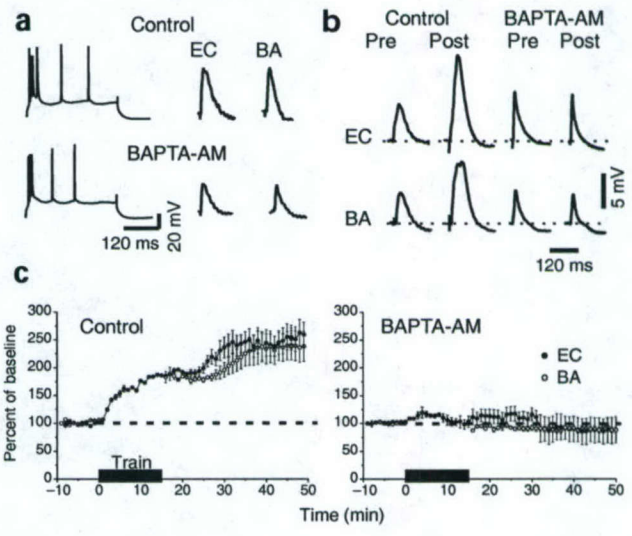


Fig. 9. Role of Ca^{2+} in low-frequency train-induced heterosynaptic facilitation. **(a)** Action potentials elicited by intracellular depolarizing current (left; 150 pA) and synaptic responses (right) evoked by external capsule (EC) and basal amygdala (BA) stimulation in a control slice and a slice where the cells were loaded with BAPTA by perfusion for 30 min with 50 μM BAPTA-AM. **(b)** Intracellularly recorded synaptic responses evoked by EC and BA stimulation before ('Pre') and 30 min after ('Post') 15 min low-frequency EC stimulation in control solution containing 100 μM APV (left) and after perfusion for 30 min with BAPTA-AM (right). **(c)** Summary of the results of experiments similar to those of **(b)** in control APV solution (left; n = 3) and after BAPTA-AM (right; n = 4). Low-frequency EC stimulation was applied during the 15-min period indicated by the bar.

Discussion

These studies have defined a form of activity-dependent, long-lasting synaptic strengthening that requires activation of GluR5 kainate receptors. The characteristics of kainate receptor-mediated enduring synaptic enhancement in the basolateral amygdala are different in many respects from conventional long-term potentiation as observed in the hippocampus, neocortex and other brain areas[1–3]. Conventional long-term potentiation occurs rapidly following a high-frequency tetanus. In contrast, enduring synaptic enhancement in basolateral amygdala is induced by prolonged low-frequency stimulation. The increase in amplitude of the synaptic response develops slowly during the stimulus train and continues to evolve even after the termination of stimulation. Unlike short-term potentiation in the basolateral amygdala and many examples of short- and long-term synaptic facilitation in other brain areas, induction of low-frequency stimulation-induced synaptic facilitation in the amygdala does not require activation of NMDA recep-

tors. However, as is the case for NMDA receptor-dependent and other forms of synaptic plasticity, Ca^{2+} influx is required.

Long-lasting, activity-dependent synaptic plasticity has been predominantly associated with a specific enhancement of the fast AMPA receptor-mediated component of synaptic transmission[1,2], although there are reports that the NMDA component can be enhanced under some circumstances[35], and this has been recently associated with metabotropic glutamate receptor activation[36,37]. Our observation that low-frequency stimulation-induced synaptic facilitation in the amygdala is eliminated by kainate receptor blockade but not by blockade of group I metabotropic receptors suggests that metabotropic glutamate receptors do not have a similar involvement in the paradigm reported here. The substrate of low-frequency stimulation-induced synaptic facilitation in basolateral amygdala neurons is yet to be defined, both with respect to the presynaptic or postsynaptic localization of the GluR5 kainate receptors that trigger the phenomenon and the underlying mechanisms that allow expression of the response. Glutamate receptors, including AMPA, NMDA and kainate receptors, could increase in number at subsynaptic sites, or their activity could be enhanced by cell factors. However, specific alterations in the synaptic localization of AMPA receptors, as in hippocampal long-term potentiation[38], is unlikely to be the sole underlying mechanism, because the non-AMPA receptor-mediated component of the synaptic response is also enhanced in a parallel fashion with the AMPA receptor-mediated component. Indeed, this suggests that presynaptic mechanisms could be involved. Inasmuch as glutamate receptors may not be saturated during ordinary synaptic transmission[39], increased neurotransmitter release could result in enhancement of the postsynaptic response. Alternatively, there could be recruitment of additional active synapses, a possibility that seems attractive given the ability of the low-frequency stimulation protocol to enhance responses from a naive convergent input. Both the homosynaptic and heterosynaptic facilitation could then occur via release of a diffusible factor that is able to act at a distance. Although our results demonstrate that low-frequency stimulation induces strengthening of the stimulated synapses in a monosynaptic fashion, there is also likely to be recruitment of multisynaptic pathways resulting in the appearance of polysynaptic responses. These polysynaptic responses could originate as a result of stimulation-induced facilitation of excitatory synaptic responses at synapses in a multi-neuron circuit, or as a result of a diffusible factor acting on such synapses. Polysynaptic responses were particularly apparent following perfusion with ATPA, which, in contrast to focal pathway stimulation, would act on GluR5 kainate receptors broadly within the slice. The spread of synaptic facilitation could mediate a general increase in excitation within the nucleus, such as might occur during epileptogenesis, a phenomenon that occurs after exposure to kainate receptor agonists[40].

The identification of kainate receptors as mediators of a form of synaptic plasticity confers an important involvement for this class of glutamate receptor. Kainate-receptor subunits are widely expressed in brain, and the GluR5 subunit is prominent in the amygdala. The availability of selective pharmacological agonists and antagonists has made it possible to demonstrate that kainate receptors mediate a component of excitatory synaptic transmission at synapses in the hippocampus, neocortex and spinal cord[41], as well as in the amygdala[8]. Until recently, these kainate receptors had not been implicated in synaptic plasticity phenomena. However, a recent report has suggested that GluR5 kainate receptors participate in the induction of mossy fiber long-term potentiation[16], although this has been questioned because of the relatively weak expression of GluR5 subunit mRNA in the dentate gyrus and CA3 regions of the hippocampus, and because some non-selective kainate receptor antagonists fail to block the potentiation[42]. In the basolateral amygdala, our studies indicate a high density of GluR5 kainate receptors. Moreover, low-frequency stimulation-induced synaptic facilitation in the amygdala is completely eliminated by GluR5 kainate receptor antagonists and can also be mimicked by application of a selective GluR5 agonist, providing strong support for the involvement of GluR5 kainate receptors in the phenomenon.

Kainate receptor responses undergo rapid and total desensitization, the recovery from which is slow[43]. This desensitizing property could, in part, contribute to the requirement for low-frequency stimulation to induce kainate receptor-mediated synaptic potentiation. Nevertheless, the optimal stimulation parameters for induction of kainate receptor-mediated synaptic plasticity are not yet fully defined. Delivery of 900 stimuli (as in our standard stimulation protocol) over one-fifth the time (5 Hz for 3 min) also results in robust [Author: As meant?] facilitation but with a latency of ~15 min following termination of the stimulation train (unpublished data). Therefore, a minimal latent interval must elapse for kainate receptor-mediated plasticity to become established, indicating that there is a rate-limiting biochemical process underlying the plasticity mechanism. The delayed onset may account for the failure to have previously detected this form of synaptic plasticity, particularly because amygdala neurons also exhibit conventional NMDA receptor-dependent forms of synaptic plasticity[44].

Analyses of the cellular mechanisms by which experience modifies behavior have, in recent years, focused on synapse-specific forms of synaptic plasticity as exemplified by hippocampal long-term potentiation in which input specificity may allow for associative learning through Hebbian synaptic mechanisms. Indeed, all examples of activity-dependent strengthening are largely input-pathway specific, although there may be potentiation of synaptic transmission at neighboring cell synapses[45]. Input specificity may not be absolute for very short distances[46], and there are examples where long-term potentiation in one population of synapses can be associated with long-term depression of neighboring, inactive synapses[47]. Kainate receptor-mediated synaptic plasticity represents a distinct form of plasticity that could play role in non-Hebbian types of behavioral adaptations. For example, in the amygdala, heterosynaptic spread of synaptic facilitation could permit diverse internal and external stimuli to activate stereotyped behavioral responses. Such mechanisms might be adaptive in life-threatening situations where similar behavioral actions are to be executed regardless of the modality through which the threat is presented, but could also contribute to pathological conditions in which amygdala-dependent behavioral states such as anxiety and fear are inappropriately triggered by innocuous stimuli, as in the posttraumatic stress syndrome[48].

Methods

Amygdala slice preparation. Male Sprague-Dawley rats weighing 75–150 g were used. The rats were decapitated, the brains rapidly removed, and 500-μm-thick transverse slices of the amygdala were cut from tissue blocks with a Vibratome (Technical Products International, St. Louis, Missouri). The slices were preincubated in oxygenated artificial cerebrospinal fluid (CSF) continuously bubbled at room temperature (23°C) with 95% O_2/5% CO_2 for at least 1 h before use. The artificial CSF contained 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose, and was bubbled with 95% O_2/5% CO_2 to maintain a pH of 7.4.

Intracellular recording. Slices were transferred to an interface chamber that was continuously superfused with artificial CSF at a rate of 1 to 2 ml/min. Microelectrodes were pulled from thin wall 1.0-mm microfiber-

filled borosilicate capillaries using a Brown-Flaming horizontal micropipette puller (Sutter Instruments, San Rafael, California). The resistance of the microelectrodes when filled with 3 M KCl or 4 M potassium acetate ranged from 80 to 150 MΩ. In some experiments, 50 mM 1,2-bis(2-amniophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA; Sigma, St. Louis, Missouri) was included microelectrode solution. The microelectrode tips were visually positioned in the basolateral region of the basal amygdala (between the EC and the stria terminalis) using a dissecting microscope. Intracellular impalements were made in a blind fashion. Recordings were terminated if the resting potential was more positive than –55 mV or if the action potential height was < 70 mV. Intracellular potentials were amplified with an Axoclamp-2A amplifier (Axon Instruments, Foster City, California; low pass filter, 3 kHz), and the output was digitized with a Digidata 1200 interface (Axon Instruments). In some experiments, a second extracellular microelectrode for field recording (1–3 MΩ when filled with 2 M NaCl) was positioned near the intracellular microelectrode. On- and off-line data acquisition and analysis was carried out using WCP version 1.7b (University of Strathclyde, Glasgow, UK).

Stimulation. Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes (World Precision Instruments, Sarasota, Florida) placed in the EC and in the BA near the accessory basal nucleus[49]. Both stimulating electrodes were ~2 mm from the recording site. Stimuli were delivered using photoelectric stimulus isolation units having a constant current output (SIU90; Stimulus Isolation Unit, Cygnus Technology, Delaware Water Gap, Pennsylvania). The stimulus intensity was adjusted to produce a synaptic response 30–50% of maximum amplitude without triggering an action potential response. Peak response amplitudes were measured with respect to the resting membrane potential. In occasional cases where action potentials were generated, for example, after synaptic potentiation, the peak amplitude of the underlying synaptic response was estimated by extrapolation. Single 0.1-ms monophasic square pulses were applied continuously throughout the experiment at 0.1 Hz (or at 0.05 Hz in experiments with dual stimulation). Such stimulation was associated with no more than a 10% drift in the peak synaptic response amplitude during experiments lasting up to 120 min. In dual-stimulation experiments, the interval between the EC and BA stimulus was 1 s. Synaptic response latencies were measured from the onset of stimulation to the time at which the synaptic response reached two standard deviations of the baseline noise. Synaptic response duration was measured from the time of stimulation to the intersection of the voltage trajectory with baseline. Data are expressed as mean ± s.e.m. Statistical comparisons were made with the *t*-test.

***In situ* hybridization.** Sense and antisense riboprobes for GluR5, GluR6 and KA2 prepared by linearizing the cDNAs in pBluescript SK(±) (donated by S. Heinemann) were labeled with [^{35}S]UTP by T3 or T7 RNA polymerases (Ambion, Texas). Brain sections (15 µm) fixed with 4% paraformaldehyde were hybridized overnight at 56°C with 1×10^6 c.p.m. of [^{35}S]UTP labeled riboprobes and exposed to Kodak Min R film together with standards for 1 to 3 days at room temperature. The sense probes did not result in detectable signals during the time of the exposure. Microphotography was performed digitally using a solid-state video camera. Brightfield images were captured with transmitted light, and mRNA expression levels in regions of interest defined by anatomical landmarks were derived from transmittance values using the Rodbard hyperbolic calibration curve of NIH IMAGE. For each structure, three sections from separate animals were measured. Right and left sides were pooled.

GluR5 RT-PCR. Total cellular RNA was extracted using RNAgents Total RNA Isolation System (Promega, Madison, Wisconsin) from discrete brain nuclei microdissected from 500-µm brain slices. The RNA content of the extracts was quantified spectrophotometrically and 2 µg of total RNA was subjected to RT-PCR using the Access RT-PCR system (Promega) with the upstream primer 5′-TTCCCCCTGCGAATAAAGACGCCA-3′ and the downstream primer 5′-CATCATGCCATCCAGAAGACCAGT-3′, along with primers for β-actin mRNA (rat β-actin control amplimer set; Clontech Laboratories, Palo Alto, California). The PCR products were analyzed by 3% agarose gel electrophoresis and staining with ethidium bromide. Quantitative analysis was done by measuring the intensity of the ethidium bromide fluorescence of each band using β-actin as an internal control.

Quantification of GluR5 editing. Total RNA (2 µg) extracted from microdissected nuclei was subjected to RT-PCR using the upstream primer 5′-GGTATAACCCCCACCCATGCAACC-3′ and the downstream primer 5′-GAAGGTCATCGTCGAGCCATCTCTG-3′. Ten microliters of the 50-µl PCR reaction product was digested with BbvI (New England Biolabs, Beverley, Massachusetts) and loaded onto 3% agarose gels along with digoxigenin-labeled DNA molecular weight marker VIII (Roche Molecular Biochemicals, Mannheim, Germany). Following electrophoresis, the DNA was transferred to 0.2 µm Nytran N nylon membranes (Schleicher & Schuell, Keene, New Hampshire) and hybridized with a probe homologous to a sequence characteristic of GluR5 splice variants (5′-CCC-GACTCAGACGTGGTGGAAAACAATTTC-3′; 24 pmol/ml) that had been 5′-end-labeled with digoxigenin. The presence of the digoxigenin-labeled probe was detected using alkaline phosphatase conjugated anti-digoxigenin antibody (1:10000 dilution) and the chemiluminescent substrate CSPD (Roche Molecular Biochemicals). Chemiluminescence was detected by exposure of the blot membrane to x-ray film for 15 to 20 min. Background subtracted optical densities were used to estimate the fraction of edited and unedited to total GluR5 mRNA.

Drugs. GYKI 53655 [1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine], LY377770 [(3S,4aR,6S,9aR)-6{[[(1H-tetrazol-5-yl)methyl]oxy]methyl}-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid] and LY382884 [(3S, 4aR, 6S, 8aR)-6-((4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid] were generous gifts of Lilly Research Laboratories, Eli Lilly and Co. (Indianapolis, Indiana). (–)-Bicuculline methochloride, APV, SCH 50911 [(+)-(2S)-5,5-dimethyl-2-morpholineacetic acid], and CPCCOEt {7-(hydroxyimino)cyclopropa[b]-chromen-1a-carboxylate ethylester} were from Tocris Cookson (Ballwin, Missouri). BAPTA acetoxymethyl ester (BAPTA-AM) was from Molecular Probes (Eugene, Oregon). BAPTA-AM was initially dissolved in DMSO as a stock solution and then diluted 1:1000 to a final concentration of 50 µM. In some cases, 0.02% Pluronic F-127 (Molecular Probes) was included in the BAPTA-AM solution.

ACKNOWLEDGEMENTS

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Lamotrigine reduces spontaneous and evoked $GABA_A$ receptor-mediated synaptic transmission in the basolateral amygdala: implications for its effects in seizure and affective disorders

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Abstract

Lamotrigine (LTG) is an antiepileptic drug that is also effective in the treatment of certain psychiatric disorders. Its anticonvulsant action has been attributed to its ability to block voltage-gated Na^+ channels and reduce glutamate release. LTG also affects GABA-mediated synaptic transmission, but there are conflicting reports as to whether inhibitory transmission is enhanced or suppressed by LTG. We examined the effects of LTG on $GABA_A$ receptor-mediated synaptic transmission in slices from rat amygdala, a brain area that is particularly important in epileptogenesis and affective disorders. In intracellular recordings, LTG (100 μM) reduced $GABA_A$ receptor-mediated IPSPs evoked by electrical stimulation in neurons of the basolateral nucleus. In whole-cell recordings, LTG (10, 50 and 100 μM) decreased the frequency and amplitude of spontaneous IPSCs, as well as the amplitude of evoked IPSCs, but had no effect on the kinetics of these currents. LTG also had no effects on the frequency, amplitude or kinetics of miniature IPSCs recorded in the presence of TTX. These results suggest that in the basolateral amygdala, LTG suppresses $GABA_A$ receptor-mediated synaptic transmission by a direct and/or indirect effect on presynaptic Ca^{++} influx. The modulation of inhibitory synaptic transmission may be an important mechanism underlying the psychotropic effects of LTG. Published by Elsevier Science Ltd.

Keywords: Lamotrigine; Amygdala; $GABA_A$; Inhibition; Affective disorders; Bipolar depression

1. Introduction

Lamotrigine (LTG) is an anticonvulsant effective in the treatment of generalized and partial seizures (Goa et al., 1993; Mikati and Holmes, 1997; Stewart et al., 1992; Timmings and Richens, 1992). The actions of LTG that are considered to be responsible for its anticonvulsant effects are: 1) blockade of voltage-gated Na^+ channels in a voltage and activity-dependent manner (Cheung et al., 1992; Xie et al., 1995), and 2) reduction of glutamate release (Calabresi et al., 1999; Cunningham and Jones, 2000; Wang et al., 1996). The inhibition of glutamate release by LTG may be mediated, in part, by an effect on Na^+ action potentials (Leach et al., 1986), or other mechanisms, such as reduction of voltage-gated Ca^{++} currents (Stefani et al., 1997), as reported in the amygdala (Wang et al., 1996; Wang et al., 1996), or by an effect on transmitter release processes downstream to Ca^{++} influx, as reported in the entorhinal cortex (Cunningham and Jones, 2000).

More recently, LTG has been found to produce beneficial results in the treatment of certain psychiatric illnesses, such as bipolar (Calabrese et al., 2000; Engle and Heck, 2000; Fogelson and Sternbach, 1997; Frye et al., 2000; Kusumakar and Yatham, 1997; Sporn and Sachs, 1997; Walden et al., 1998; Weiss and Post, 1998) and unipolar (Frye et al., 2000) mood disorders, and possibly schizophrenia (Dursun et al., 1999; Erfurth et al., 1998). The mechanisms mediating the psychotropic effects of LTG are unknown. Although the dampening of overexcited neuronal networks by an effect on Na^+ channels and glutamate release could be involved in some of the psychotropic effects of LTG (Xie and Hagan, 1998),

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other actions of the drug deserve closer attention. Several studies suggest that LTG suppresses GABA-mediated synaptic transmission. Thus, LTG reduces GABA release evoked by electrical stimulation in rat spinal dorsal horn slices (Teoh et al., 1995), or by the Na^+ channel activator veratrine in rat cortical slices (Leach et al., 1986; Waldmeier et al., 1995). LTG also suppresses spontaneous, inhibitory synaptic events in cultured rat cortical neurons (Lees and Leach, 1993). However, in slices from rat entorhinal cortex, LTG was found to increase both spontaneous and miniature inhibitory potentials (Cunningham and Jones, 2000). In view of the important role of the neurotransmitter GABA not only in epilepsy, but also in the pathophysiology of mood disorders (for a review see Petty, 1995; Squires and Saederup, 1991), we investigated how LTG affects $GABA_A$ receptor-mediated synaptic transmission in the amygdala, an area of the brain that plays an important role in the pathophysiology of epileptogenesis and affective disorders (Aggleton, 2000). A better understanding of the mechanisms accounting for the anticonvulsant and psychotropic effects of LTG may provide a basis for more efficacious treatments of these disorders.

2. Methods

Coronal slices containing the amygdala were prepared from 15–22 day-old, Sprague Dawley rats. The rats were lightly anesthetized with halothane and then decapitated. The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 117 NaCl, 4.7 KCl, 1.2 $CaCl_2$, 2.5 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose, bubbled with 95% O2/5%CO_2 to maintain a pH of 7.4. A block containing the amygdala region was prepared by rostral and caudal coronal cuts, and 400 μm thick slices were cut using a Vibratome (series 1000, Technical Products International, St. Louis, Missouri). Slices were kept in a holding chamber at room temperature. Recordings were initiated 1–2 hours after slice preparation.

For intracellular recordings, the slices were transferred to an interface chamber maintained at 33°C. Slices were perfused with ACSF composed of (in mM) 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 MgCl2, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose, at a rate of 0.7 ml/min. Neurons were recorded in the basolateral nucleus of the amygdala (BLA) using glass pipettes (90–120 MΩ resistance) filled with 4 M potassium acetate. Synaptic responses were evoked with a twisted, bipolar stimulating electrode made from 50 μm–diameter stainless-steel wire. Signals were amplified with the Axoclamp 2B amplifier, used in the bridge mode, filtered at 10 kHz and digitized on line at 5 kHz.

For whole-cell recordings, slices were transferred to a submersion-type recording chamber where they were continuously perfused with oxygenated ACSF (composition as in intracellular recordings), at a rate of 1–2 ml/min. Neurons were visualized with an upright microscope (Nikon Eclipse E600fn) using Nomarski-type differential interference optics through a 60X water immersion objective. All experiments were carried out at 27–30°C. Drugs were applied by gravity, switching between four perfusion lines. Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes (World Precision Instruments, Sarasota, Florida) placed in the BLA 100–200 μm near the recording electrode (see Fig. 1A). Stimuli were delivered using a photoelectric stimulus isolation unit having a constant current output (PSIU6, Grass Instrument CO., W. Warwick, RI). Single, 0.15 ms monophasic square pulses were applied continuously throughout the experiment at 0.1 Hz. Tight-seal (>1 GΩ) whole cell recordings were obtained from the cell body of neurons in the BLA region. Patch electrodes were fabricated from borosilicate glass and had a resistance of 1.5–5.0 MΩ when filled with a solution containing (in mM): EGTA, 10; HEPES, 10; CsCl, 160; $MgCl_2$, 1; NaATP, 2; Na_3GTP, 0.2 (pH 7.3, 285–290 mOsm). Neurons were voltage clamped using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Currents were recorded at a holding potential of −80 mV (except for one cell that was recorded at the holding potential of −60 mV). Because of the presence of a chloride-rich solution in the recording pipette, $GABA_A$-mediated inhibitory currents were recorded as inward currents at these potentials (Fig. 1B). Access resistance (7–25 MΩ) was regularly monitored during recordings, and cells were rejected if it changed more than 15% during the experiment. The sig-

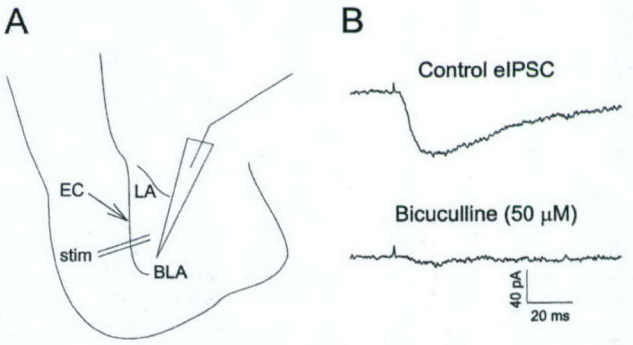

Fig. 1. A: Schematic representation of the amygdala slice showing the position of the stimulating and recording electrodes. Recordings were obtained from neurons in the BLA. The stimulating electrode was placed very close to the recording pipette, in whole-cell recordings (see Methods), or somewhat farther, and closer to the LA, in intracellular recordings. BLA, basolateral amygdala; LA, lateral amygdala; EC, external capsule. B: Inhibitory postsynaptic currents were recorded from visually identified, BLA pyramidal neurons in response to BLA stimulation. At a holding potential of −80 mV, evoked inhibitory currents (eIPSCs) were inward due to the symmetrical intra- and extracellular concentrations of chloride. Evoked IPSCs were completely blocked by bath application of bicuculline (50 μM).

nals were filtered at 2 kHz, digitized (Digidata 1322A, Axon Instruments, Inc.), and stored on a computer using the pCLAMP8 software (Axon Instruments, Inc.). The peak amplitude, 10–90% rise time, and decay time constant of IPSCs were analyzed off-line using the pCLAMP8 software (Axon Instruments) and the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). All data are presented as mean±SEM. Results were tested for statistical significance using the Student's paired *t*-test.

The following drugs were used: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a kainate/AMPA receptor antagonist (Research Biochemicals Int.), D-2-amino-5-phosphonovalerate (APV), an NMDA receptor antagonist (Research Biochemicals Int.), CGP-35348 or SCH50911, $GABA_B$ receptor antagonists (Tocris), tetrodotoxin (TTX), a sodium channel blocker (Sigma), bicuculline methchloride, a $GABA_A$ receptor antagonist (Sigma), and lamotrigine (gift from Glaxo Wellcome Foundation). To prepare stock solutions, APV, CGP–35348, SCH50911, TTX and bicuculline methchloride were dissolved in dH_2O, whereas CNQX and lamotrigine were dissolved in DMSO (final concentration of DMSO in the slice medium was 0.01 to 0.04%, v/v).

3. Results

3.1. Effects of LTG on evoked IPSPs

Intracellular recordings were obtained from neurons in the basolateral nucleus of the amygdala (see Fig. 1A). Single pulses or paired–pulse stimulation (two successive pulses delivered at a short interstimulus interval) were applied at 0.1 Hz to a site above the location of recording, close to the lateral nucleus. $GABA_A$ receptor-mediated IPSPs were recorded in the presence of 20 μM CNQX, 50 μM APV, and 500 μM CGP–35348, to block kainate/AMPA, NMDA, and $GABA_B$ receptors, respectively.

Paired-pulse stimulation produces depression (paired-pulse depression, PPD) or facilitation (PPF) of the synaptic response to the second pulse. An important factor determining whether PPD or PPF will be produced is the probability of neurotransmitter release during the first pulse. A high probability of neurotransmitter release in response to the first pulse will result in PPD, whereas a low probability of neurotransmitter release in response to the first pulse will result in PPF (Bertrand and Lacaille, 2001; Debanne et al., 1996; Jiang et al., 2000; Manabe et al., 1993; Thomson et al., 1993). The IPSPs in the BLA displayed a small PPD when paired pulses were delivered at an interstimulus interval of 80 msec (Fig. 2). Bath application of 100 μM LTG reduced the amplitude of the first IPSP by 47±7.03% (n=5). LTG also reduced PPD of the IPSPs (n=2), reversing PPD to PPF in some cells (n=3) (Fig. 2). Ratio measurements of the second to the first IPSP amplitude were made in those traces where the IPSP evoked by the first pulse had returned to baseline before the arrival of the second pulse; this ratio was 0.84±0.01 in control conditions, and 1.12±0.1 in the presence of LTG ($P<0.05$, n=5). These results suggest that the reduction of the IPSPs by LTG could be due to a reduction in the probability of presynaptic GABA release.

LTG had no significant effects on resting membrane potential or input resistance of the recorded neurons. The effects of LTG on the IPSPs were reversible. With our flow rate in the interface chamber (0.7 ml/min, see Methods) it took 10–15 min of wash-in for LTG to take full effect, and 30–35 min of washing-out for the effects to be reversed.

3.2. Effects of LTG on IPSCs

Whole-cell recordings from visualized pyramidal cells in the BLA were obtained at the holding potential of −80 mV (unless stated otherwise). Inhibitory postsynaptic currents (IPSCs) were recorded in the presence of 20 μM CNQX, 50 μM APV, and 10 μM SCH–59011. First, we examined the effects of LTG on spontaneous IPSCs (sIPSCs). Spontaneous IPSCs are triggered, primarily, by spontaneous action potentials invading GABAergic presynaptic terminals, where they trigger Ca^{++} influx and GABA release. Therefore, the frequency and amplitude of these currents are sensitive to blockade of voltage activated Na^+ or Ca^{++} channels. Bath application of 100 μM LTG reduced the frequency and amplitude of sIPSCs, with no effect on rise time or decay time constant (Fig. 3B). The interval between spontaneous events was increased by 58±3.2% ($P<0.01$, n=7) in the presence of LTG. The frequency of sIPSCs was reduced from 5.2±0.41 Hz to 2.3±0.34 Hz, while the amplitude was reduced by 71±2.8 % ($P<0.01$, n=7). Similar results were obtained with 10 μM LTG (Fig. 3A). A somewhat longer time period of wash-in was required before LTG takes effect at this concentration, and the effect was smaller. Thus, at 10 μM, LTG increased the interval between spontaneous IPSCs by 29±2.7% ($P<0.01$, n=7), and reduced the amplitude of the IPSCs by 35±5.7% ($P<0.01$, n=7).

Next, we examined the effects of LTG on IPSCs evoked by electrical stimulation. At 100 μM, LTG decreased the amplitude of evoked IPSCs (eIPSCs) by 91±2.5% ($P<0.01$, n=7, Fig. 4). At 50 and 10 μM LTG, the amplitude of IPSCs was reduced by 56±4.7% ($P<0.01$, n=7) and 31±5.4% ($P<0.01$, n=7), respectively (Fig. 4). The kinetics of the IPSCs were not altered (Fig. 4C). Thus, the 10–90% rise time and decay time constant were 2.1 ± 0.8ms and 9.4±1.1ms before, and 2.2±1.0ms and 9.8±1.3 ms in the presence of LTG, respectively. All of the effects of LTG were reversible.

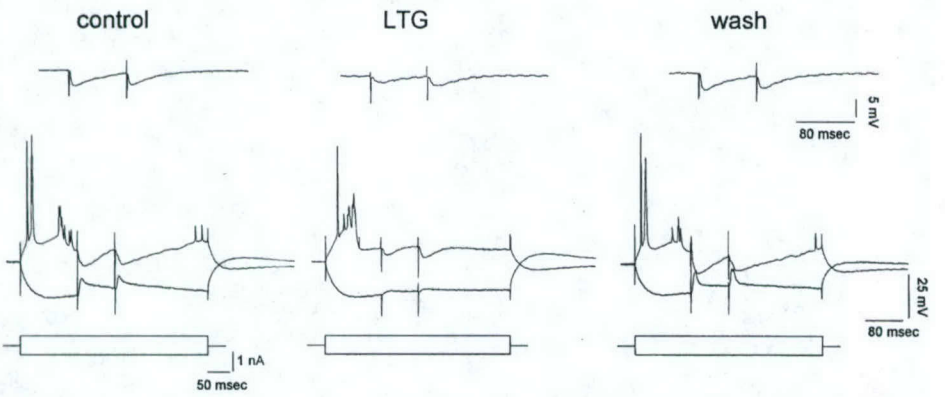

Fig. 2. Effects of LTG on evoked IPSPs. All traces are recordings from the same cell. The top row shows IPSPs recorded at resting membrane potential (−69 mV). Paired-pulses are delivered at 80 msec inter-pulse interval. LTG (100 μM) reversibly reduced the evoked IPSPs, and reversed paired-pulse depression to a small facilitation. Bottom row shows IPSPs evoked by paired-pulse stimulation applied during 400 msec hyperpolarizing (−0.5 nA) or depolarizing (+0.5 nA) intracellular current pulses. The IPSPs increased during depolarization, and reversed polarity during hyperpolarization as expected for chloride-mediated potentials. Each trace is an average of five to ten sweeps (the variable shape and size of the spikes is due to averaging).

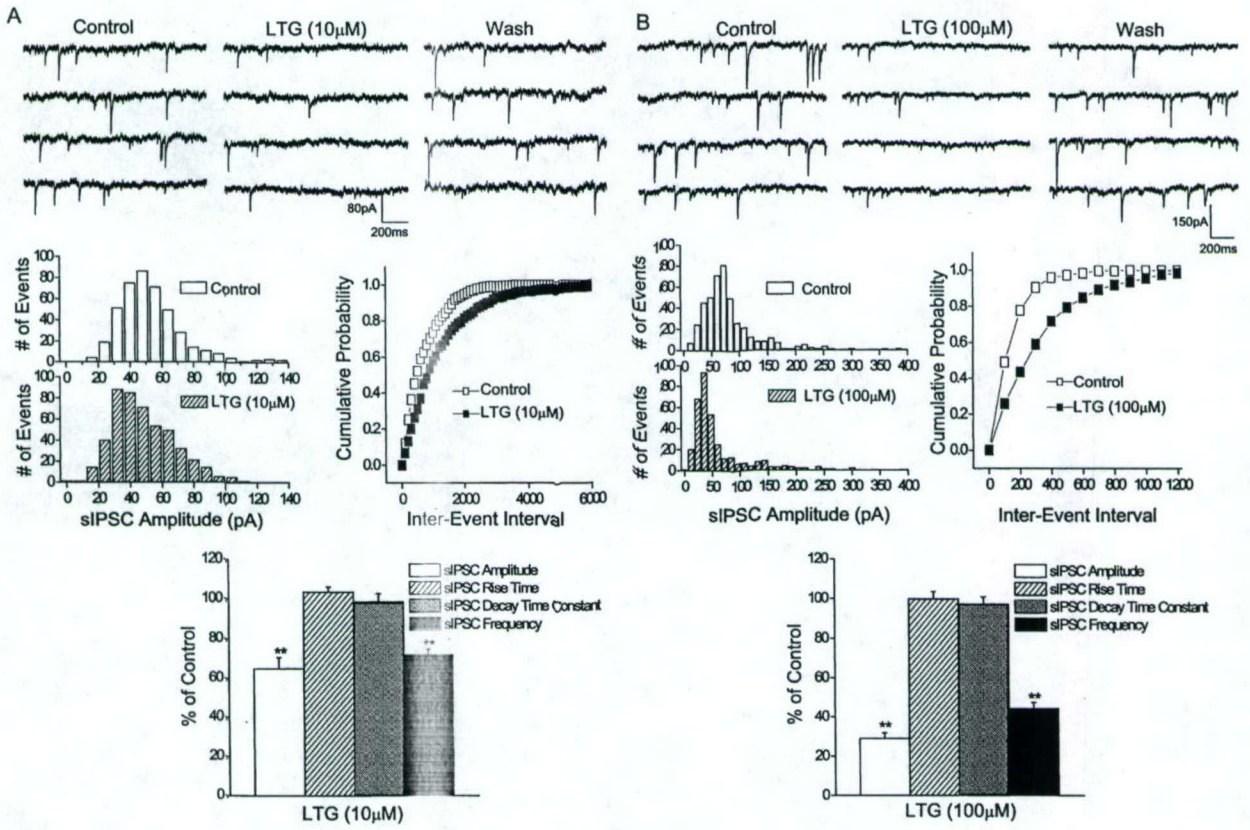

Fig. 3. Effects of LTG on spontaneous IPSCs. Top traces: Samples of sIPSCs recorded from two neurons before, during, and after perfusion with external solution containing 10 μM (A, Holding potential = −60 mV) or 100 μM (B, Holding potential=−80 mV) LTG. Both the amplitude and the frequency of the currents were reduced by LTG. Middle graphs: Amplitude histograms of sIPSCs and cumulative probability plots of inter-event intervals in control conditions and during LTG perfusion (same cells as in the top). LTG at 10 and 100 μM reduced the amplitude of the sIPSCs and increased the inter-event distribution. The magnitude of these effects was dependent on the concentration of LTG. Bottom graphs: Vertical bars and error bars represent the mean±SE of results obtained from seven neurons. The peak amplitude, rise time, decay time constant and frequency of sIPSCs recorded in the presence of LTG were calculated as a percentage of the control values. $**P<0.01$ (t-test).

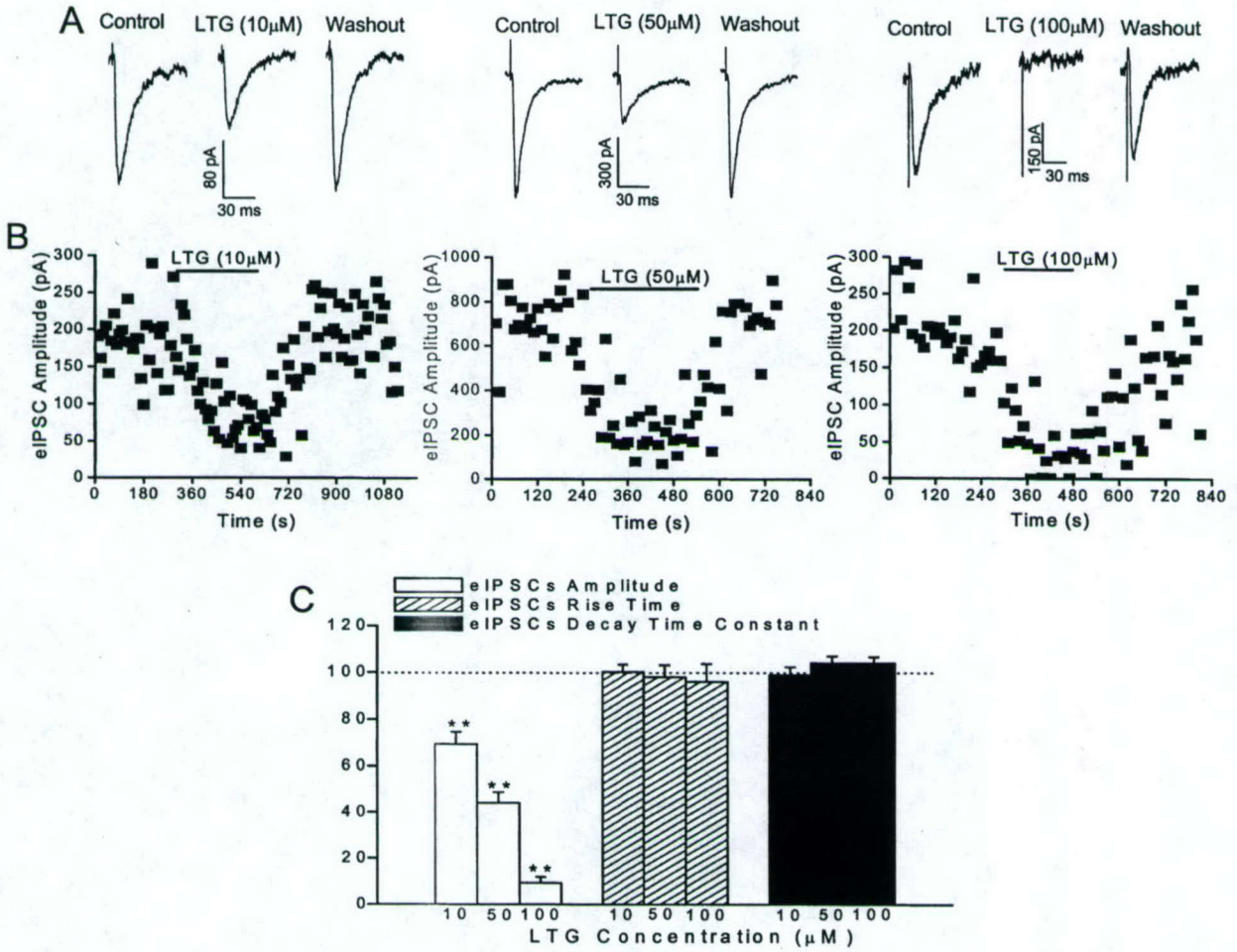

Fig. 4. Effects of LTG on evoked IPSCs. A: Samples of eIPSCs recorded from three different neurons before, during, and after perfusion with external solution containing 10, 50 or 100 μM LTG. The amplitude of the currents were reversibly reduced by LTG in a concentration dependent manner. Holding potential, −80 mV. B: Plots showing the time course of the effects of LTG on the amplitude of eIPSCs (same cells as in A). C: Vertical bars and error bars represent the mean±SE of results obtained from seven neurons. The peak amplitude, rise time and decay time constant of eIPSCs recorded in the presence of LTG were calculated as a percentage of control (in absence of LTG) values. LTG had no effect on the rise time and decay time constant of the eIPSCs, while it reduced significantly their amplitude. $**P<0.01$ (t-test).

In two cells we re-applied LTG after the first wash-out, and the same effects were reproduced (data not shown).

The results described above are consistent with a presynaptic mechanism of LTG action, but do not exclude concomitant postsynaptic effects. To confirm that LTG did not act postsynaptically we examined its effects on miniature IPSCs (mIPSCs). mIPSCs were recorded in medium containing the same drugs as described above, with the additional presence of 1 μM TTX. Miniature currents do not depend on presynaptic action potentials and Ca^{++} influx; therefore, a change in their amplitude would indicate postsynaptic changes, whereas a change in their frequency would indicate changes in neurotransmitter release processes downstream to Ca^{++} influx. The frequency of mIPSCs recorded in the soma of BLA pyramidal neurons was 1.9±0.51 Hz, while the amplitude was 57±12 pA (n=6). LTG at 100 μM had no effect on the frequency, amplitude, or the kinetics of mIPSCs (n=6, Fig. 5). The lack of an effect on the amplitude and kinetics of the mIPSCs suggests that LTG had no postsynaptic effects. The lack of an effect of LTG on the frequency of mIPSCs suggests that LTG does not interfere with presynaptic events downstream to Ca^{++} influx.

4. Discussion

Previous studies have suggested that, in addition to inhibiting glutamate release, LTG also inhibits GABA release. Thus, in rat spinal–dorsal horn slices, LTG reduced the amount of GABA released in the slice medium following repetitive electrical stimulation (Teoh et al., 1995). In rat cerebral cortical slices, LTG reduced veratrine-evoked GABA release in the slice medium (Leach et al., 1986; Waldmeier et al., 1995). In addition,

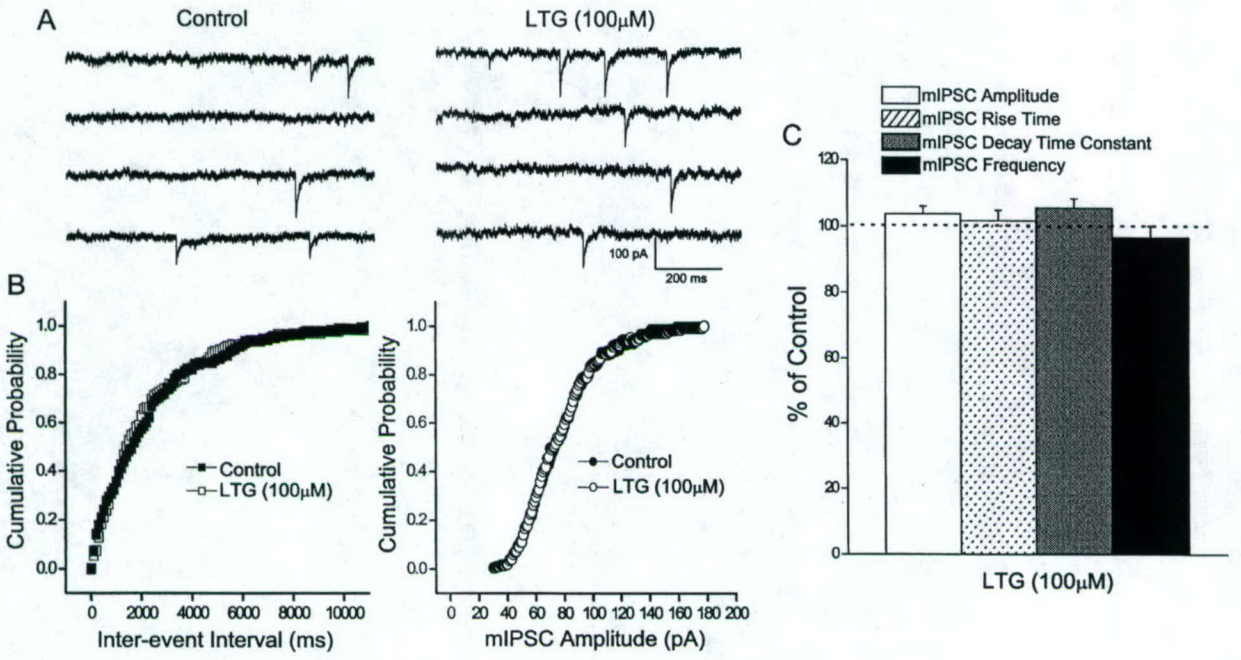

Fig. 5. LTG has no effect on the frequency, amplitude, rise time and decay time constant of miniature IPSCs recorded from BLA neurons. Spontaneous mIPSCs were recorded in the presence of TTX (1 μM). A: Samples of miniature IPSCs recorded before and during perfusion of a neuron with external solution containing LTG (100 μM). Holding potential, −80 mV. B: Cumulative probability plots of inter-event intervals and amplitude of mIPSCs in control conditions and during LTG perfusion. C: Vertical bars and error bars represent the mean±SE of results obtained from six neurons. The peak amplitude, rise time, decay time constant and frequency of mIPSCs recorded in the presence of LTG were calculated as a percentage of the control values. LTG produced no significant changes on any of these parameters.

in cultured cortical neurons, LTG decreased the frequency of spontaneous inhibitory events (Lees and Leach, 1993). The present findings are consistent with these studies, and provide the first, solid evidence that LTG reduces $GABA_A$ receptor-mediated synaptic transmission in intact circuits of the basolateral amygdala.

The site of LTG action appears to be at GABAergic presynaptic terminals rather than postsynaptically on $GABA_A$ receptors, or other postsynaptic conductances. This conclusion is supported by the following findings: 1) In intracellular recordings, the reduction of IPSPs by LTG was not accompanied by postsynaptic changes in the resting membrane potential or input resistance of the recorded neurons. Furthermore, there was a small but significant effect on paired-pulse plasticity (reduction of PPD or its reversal to PPF), which suggests a reduction in the probability of presynaptic GABA release. 2) In whole-cell recordings, LTG decreased the frequency and amplitude of spontaneous IPSCs, as well as the amplitude of evoked IPSCs. Although these effects could, in part, be mediated by a postsynaptic action of LTG, this possibility can be ruled out because the amplitude of miniature IPSCs was not affected by LTG. Thus, LTG reduces inhibitory synaptic transmission in the BLA by a presynaptic effect on GABA release.

Presynaptically, LTG can reduce the frequency of spontaneous GABA release as well as the amplitude of spontaneous or evoked GABA release either by reducing Ca^{++} influx into GABAergic terminals (directly or via inhibition of Na^+ channels, see below), or by interfering with GABA release processes downstream to Ca^{++} influx. The latter possibility is unlikely because the frequency of miniature IPSCs, which do not depend on presynaptic action potentials and Ca^{++} influx, was not affected by LTG. Thus, the results suggest that LTG reduced Ca^{++} influx into the presynaptic terminals. This effect could be either direct via modulation of voltage-dependent Ca^{++} channels, or indirect via suppression of presynaptic Na^+ action potentials. Inhibition of glutamate release by LTG in the amygdala, at concentrations of LTG similar to those used in the present study, is mediated, at least in part, by N-type calcium channels (Wang et al., 1996a,b). Whether LTG affects the same type of calcium channels on GABAergic terminals of the amygdala remains to be determined.

In a recent study in slices from rat entorhinal cortex, it was found that LTG, at the same concentrations as those used in the present study, enhanced the frequency and amplitude of spontaneous IPSCs, and the frequency of miniature IPSCs (Cunningham and Jones, 2000). It was concluded that LTG increases GABA release by a mechanism downstream to calcium influx. The discrepancy between these observations and our present results might be due to the different brain area under investigation.

Previous studies reporting a reduction of GABA release by LTG found that higher LTG concentrations were needed for an effect on GABA release compared to the effect on glutamate release (Leach et al., 1986; Teoh et al., 1995). For this reason, it was suggested that the principal effect of LTG is on glutamate release, while the reduction of GABA release did not receive much attention as a clinically relevant finding. In the present study however, LTG inhibited $GABA_A$-receptor mediated transmission even at concentrations similar to those found in the blood of LTG-treated human patients (10–15 μM; Messenheimer, 1995), suggesting that the effect may be clinically relevant. It is possible that reduction of excessive firing and glutamate release are indeed the principal effects of LTG in hyperexcited, epileptic neuronal networks, whereas in normal or hypoactive (overinhibited) networks other effects of LTG become also important. There is already evidence supporting this possibility: LTG suppresses kindling-induced, fully-developed seizures in the amygdala, but, depending on the dose, it has no effect or facilitates the development of these seizures (Postma et al., 2000). The present results suggest that reduction of inhibitory transmission could be one of the mechanisms by which LTG facilitated the development of amygdala kindling.

LTG has produced promising results in certain psychiatric diseases where amygdala dysfunction is believed to play a central role, such as bipolar (Calabrese et al., 2000; Engle and Heck, 2000; Fogelson and Sternbach, 1997; Frye et al., 2000; Kusumakar and Yatham, 1997; Sporn and Sachs, 1997; Walden et al., 1998; Weiss and Post, 1998) or unipolar depression (Frye et al., 2000). LTG may also be effective in treatment-resistant schizophrenia (Dursun et al., 1999; Erfurth et al., 1998). Because an overactive GABAergic system may significantly underlie some types of these disorders (Squires and Saederup, 1991), the effectiveness of LTG may be due, in part, to its ability to suppress inhibition. As both spontaneous and evoked inhibition were found to be reduced by LTG, treatment with this compound could suppress overactive GABAergic inhibition both at the basal state and when neuronal circuits are active during synaptic processing.

The dual effect of LTG on suppressing both glutamatergic and GABAergic activity in the amygdala seems suitable for suppression of sequentially overactivated excitatory and inhibitory systems, as it appears to occur in bipolar depression. These neurotransmitter systems are also key players in synaptic plasticity (Aroniadou–Anderjaska et al., 2001; Li et al., 2001; Rammes et al., 2000). Whether LTG affects amygdala synaptic plasticity remains to be determined.

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